# PURIFICATION AND CHARACTERIZATION OF GALACTOSE BINDING LECTIN FROM *ARTOCARPUS HIRSUTA*

A THESIS SUBMITTED TO THE
**UNIVERSITY OF PUNE**
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DEDICATED TO
MY PARENTS

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## Declaration

Certified that the work incorporated in the thesis entitled "Purification and Characterization of galactose binding lectin from *Artocarpus hirsuta*" submitted by Ms Madhura M. Gurjar was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. M. I. Khan
Research Guide

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# ABSTRACT

Lectins are carbohydrate binding proteins found in plants, animals and micro-organisms. They find application in 1] isolation of glycoproteins 2] structural studies of carbohydrates 3] investigation of protein-carbohydrate interactions 4] identification of cell surface sugars and 5] detection of cell surface changes during differentiation, development and malignancy. The biological role of lectins is a consequence of their binding to the cell surface carbohydrates.

*Artocarpus* is a tropical plant from the family Moraceae. Jacalin, the lectin from *Artocarpus integrifolia* and the lectin from *A. lakoocha* are specific for the tumor associated T antigen. The potential applications of *Artocarpus* lectins make them an ideal candidate for study. Another *Artocarpus* species namely, *A. hirsuta* showed the presence of a lectin with a high titre. Investigation was carried out to purify and characterize this lectin, to determine its structure-function relationship and potential applications. The thesis is divided into five chapters.

**Chapter I : General Introduction**

This part comprises a literature survey of lectins with reference to their purification, properties and applications.

**Chapter II : Purification and characterisation of *Artocarpus hirsuta* lectin.**

The lectin was purified to homogeneity by ammonium sulphate precipitation, negative adsorption on DEAE-cellulose followed by CM-Sephadex chromatography. It was also purified by affinity chromatography on guar gum. The purified lectin is a tetramer of molecular mass 60,000, consists of two pairs of non-identical subunits of Mr 15,800 and 14,200 and has a pI of 8.5. It is a glycoprotein and contains 6.25% neutral sugar. It agglutinates rabbit and human erythrocytes, does not require metal ions for

activity and is stable over a wide range of pH (3-9). However it shows low temperature stability and loses 25% activity above 40°C within two hours.

Hemagglutination inhibition studies indicated that among the monosaccharides, galactose is a better inhibitor of *A. hirsuta* lectin than glucose and mannose. The inability of glucose and mannose to inhibit the lectin activity can be attributed to the inversion of the C-4 hydroxyl group while the non-inhibitory nature of fucose indicated the importance of the hydroxyl group at C-6. The hydroxyl group at C-2 had little influence on sugar binding to the lectin as affinities for galactose, galactosamine and 2-deoxy galactose were similar. Methyl α-galactose was found to be the most potent inhibitor of the lectin.

The fluorescence of methylumbelliferyl α-galactose is totally quenched on binding to *A. hirsuta* lectin with no change in the emission maximum. This quenching could be reversed on addition of the methyl α-galactose. Scatchard analysis of the quenching data revealed the presence of two sugar binding sites per molecule of *A. hirsuta* lectin.

**Chapter III : Chemical modification studies on *Artocarpus hirsuta* lectin**

Chemical modification studies on purified *A. hirsuta* lectin revealed the involvement of lysine and tyrosine in the sugar binding. Detailed investigation revealed that the positive charge of the ε–amino group of lysine and the phenyl ring of tyrosine are important for the sugar binding activity of the lectin. These studies also showed that tryptophan, carboxylate, histidine, serine and arginine residues have no role in the sugar binding activity.

**Chapter IV : Fluorimetric studies on *Artocarpus hirsuta* lectin**

The binding of *A. hirsuta* lectin to methyl α-galactose resulted in enhancement of the intrinsic fluorescence intensity of the lectin without any

change in the emission maximum (333 nm). The association constant for methyl β-galactose was lower than that of galactose, which can be attributed to the equatorial orientation of the C-1 methyl group.

The association constants for these sugars, decreased with increase in temperature. The enthalpy values calculated for methyl α-galactose, galactose and methyl β-galactose from the van't Hoff plots were similar and in the range -50 to -55 $kJmol^{-1}$. The difference in their affinities reflects the difference in the entropy. ΔS values for methyl α-galactose, galactose and methyl β-galactose were -85$Jmol^{-1}K^{-1}$, -111$Jmol^{-1}K^{-1}$ and-150$Jmol^{-1}K^{-1}$ respectively. Methylumbelliferyl α-galactose showed the highest affinity and a relatively positive entropic value of -13.2 $Jmol^{-1}K^{-1}$. The increased affinities of methylumbelliferyl galactose and methyl α-galactose over galactose and methyl β-galactose could be due to apolar interactions of the methylumbelliferyl group and methyl group with a hydrophobic pocket at/near the sugar binding site. Solute quenching studies on *A. hirsuta* lectin using acrylamide, potassium iodide and cesium chloride, carried out in the absence and presence of methyl α-galactose, showed that out of the four tryptophan residues in the lectin one is on the surface and accessible to the ionic quenchers. The remaining three tryptophans are buried and accessible only to the neutral quencher.

**Chapter V : General Discussion and Conclusions**

This part compares the properties of *A. hirsuta* lectin with other plant lectins.

**Appendix I : Affinity chromatography on immobilised *Artocarpus hirsuta* lectin**

This section describes the immobilisation of the basic lectin on Sepharose 4B and its potential application as an analytical tool.

## Appendix II : Characterisation of a variant lectin from *Artocarpus hirsuta*

During the course of purification of the α-galactoside binding lectin from *A hirsuta*, it was noted that approximately 30-40% of the activity bound to DEAE-cellulose while the remaining activity came in the flow through fractions. Characterisation of the bound lectin showed that its physical properties were different from that of the major lectin fraction but other properties namely sugar specificity and amino acid residues involved in the sugar binding site were the same, suggesting that this lectin could be a variant of the basic lectin.

## LIST OF ABBREVIATIONS USED

1. Fuc : L-Fucose
2. Gal : D-Galactose
3. GalNAc : N-acetyl-D-galactosamine
4. Glc : D-Glucose
5. Man : D-Mannose
6. Me : Methyl
7. MeUMb : Methylumbelliferyl
8. pNP : Paranitrophenyl
9. RCA : *Ricinus communis* agglutinin
10. WBA : Winged bean agglutinin
11. WGA : Wheat germ agglutinin
12. PHA : Phytoagglutinin from Red Kidney bean
13. THS : Tris Buffer Saline
14. PBS : Phosphate Buffer saline
15. IEF : Isoelectric focussing
16. PAGE : Polyacrylamide Gel Electrophoresis
17. DEP : Diethylpyrocarbonate
18. DTNB : Dithiobisnitrobenzoic acid
19. HNBB : Hydroxy nirobenzyl bromide
20. NBS : N-bromosuccinimide
21. PMSF : Phenylmethylsulphonylfluoride
22. NAI : N-acetylimidazole
23. TNM : Tetranitromethane
24. WRK : Woodward's reagent K
25. $K_a$ : Affinity constant
26. $K_q$ : Modified Stern Volmer quenching constant

# CHAPTER I

# GENERAL INTRODUCTION

## HISTORY OF LECTINS

Stillmark in 1888 [1] was the first to describe the phenomenon of hemagglutination by plant extracts. He found that castor beans contain a toxic protein, ricin, which agglutinated human and animal red cells. Soon after Hellin discovered abrin [2], the toxic protein from jequirity beans. Ehrlich, during the 1890's [3,4] worked with ricin and abrin to discover some of the fundamental principles of immunology namely, immunospecificity and reversibility of the antigen-antibody reaction. While investigating the properties of lectins, Landsteiner and Raubitschek in 1908 [5] established that lectins show specificity for the red blood cells of certain species. In 1945, Boyd and Reguera [6] discovered that agglutinins are also blood group specific. Crystallisation of ricin was accomplished in the late 1940's [7]. One of the first lectins to be crystallised was concanavalin A from jackbean by Sumner [8]. In 1952, Watkins and Morgan found that the lectin activity was sugar specific and hemagglutination could be inhibited by specific sugars [9].

## DEFINITION

The term "lectin" initially referred to the ability of some plant seed extracts to distinguish between human blood groups by selective agglutination (from the Latin word *legere*, which means "to select"). This term was widely applied to proteins showing agglutination, hence the term agglutinin came to be used. Goldstein et al. [10] defined lectins as sugar binding proteins or glycoproteins of non-immune origin which agglutinates cells and/or precipitates glycoconjugates.

Since the initial discovery by Stillmark in 1888 [1], several lectins have been characterised and it was found that lectins from different sources differ in their molecular structure and specificity with the common link between them being their ability to specifically and reversibly bind

carbohydrates. Hence, Peumans and Van Damme [11] proposed that plant lectins be defined as plant proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligo-saccharide. They classified lectins into three types based on their overall structure (Fig I.1).

## CLASSIFICATION

***Merolectins:*** These are small single polypeptide proteins, which because of their monovalent nature are incapable of precipitating glycoconjugates or agglutinating cells. Examples of this group are Hevein [12] and the monomeric mannose binding protein from orchids, which is very similar to the dimeric mannose specific lectin from the same species except that they occur as monomers [13].

***Hololectins:*** These contain two or more carbohydrate binding domains that are identical or very homologous. This group comprises of lectins with two or more binding sites and is capable of agglutinating cells. Majority of plant lectins fall in this category.

***Chimerolectins*:** These are fusion proteins possessing a carbohydrate binding domain along with an unrelated domain having some other well defined biological activity that acts independently of the other domain. Depending on the number of binding sites, chimerolectins behave as merolectins or hololectins. For example, type 2 ribosome-inactivating proteins (RIP's) like ricin with two carbohydrate binding sites on their B chain agglutinate cells whereas class I chitinases with a single chitin binding domain do not [11].

Therefore, any protein with a carbohydrate-binding domain may be described as a lectin. Lectins can be distinguished from glycoproteins in that, in the latter sugar is covalently bound while in lectins (which may be glycoproteins) the sugar is bound through noncovalent interactions. Lectins also differ from antibodies in that they are of non-immune origin,

constitutive, structurally diverse and specific for carbohydrates. Antibodies in contrast are immune in origin, produced in response to foreign stimulus, structurally similar and specific for nucleic acids, proteins and/or carbohydrates.

## DETECTION

Lectins have generally been detected by hemagglutination [14]. The erythrocytes used (human or animal) may be plain or treated with papain, trypsin or neuraminidase [15]. Other types of cells [14] and polysaccharides [16] have also been used for the detection of lectins. Techniques like affinity electrophoresis [17,18] and enzyme multiplied assay [19] too have been employed.

## OCCURRENCE

***Vertebrate lectins*** : These belong to two classes depending on their solubility. (i) Integral membrane lectins which require detergents for their extraction viz, galactose, mannose/ N–acetyl glucosamine specific receptors for mammalian as well as avian hepatocytes [20] and (ii) soluble vertebrate lectins namely β-galactoside specific lectin from the electric organ of the eel *Electricus electricus* [21] and the L-fucose specific lectin from the serum of the eel *Anguilla rostrata* [22] which do not require detergents for their extraction.

A recent classification indicates that most animal lectins fall into one of the five major groups: the C type or $Ca^{++}$ dependant lectins, galectins [23,24], the P type Man 6–$PO_4$ receptors, the I type lectins including sialoadhesins and immunoglobin like sugar binding proteins and the L type lectins related in sequence to the leguminous plant lectins [25].

***Invertebrate lectins:*** These are mainly found in the haemolymph and sexual organs, eg albumin glands and eggs [26,27]. Most of these lectins

are specific for sialic acid, with the exception of blood type H specific *Helix pomatia* lectin [28].

***Protozoa*:** The occurrence of lectins in lower animals, protozoa, has been best documented in pathogenic amoeba *Entamoeba histolytica*. Two distinct lectins, one specific for β1-4 linked oligomers of N-acetyl glucosamine [29] and the other specific for galactose and N-acetyl galactosamine [30] have been isolated from *E. histolytica*.

***Microbial lectins***: Many bacterial species have the ability to produce surface lectins. In Enterobacter (*Escherichia coli* and *Salmonella* species), the lectins are commonly found in the form of submicroscopic hair like appendages known as fimbriae (pili) that protrude from the cell surface. Fimbriae are usually 5-7 nm in diameter and 100-200 nm in length. Purified fimbriae consist of several hundred fimbrillin subunits. Most fimbriae are resistant to detergents and other chaotropic agents. The carbohydrate binding sites of P, S and type I fimbriae reside in the minor subunit constituents with molecular sizes of 28-35 kDa located at the tips of the fimbriae and at large intervals along their length [31]. Type I (mannose specific) fimbriae of *E. coli* preferentially bind oligomannose and hybrid oligosaccharides of animal cell surface glycoprotein [32]. P type fimbriae, also of *E. coli,* interacts specifically with glycolipids containing Gal α 1-4 Gal [33]. The S type fimbriae of *E. coli* are specific for Neu Ac α 2-3 Gal [34] whereas Type 2 fimbriae of oral actinomyces are specific for Gal β1-3 GalNAc [35].

Fungal lectins have also been studied. Majority of the lectins purified are from the fruiting bodies of Basidiomycetes, especially *Agaricus bisporus* (the commercial mushroom) [36]. Sugar specificity of fungal lectins varies from simple monosaccharides to complex carbohydrates. *Kluyveromyces bulgaricus* lectin is specific for galactose and N-acetyl

glucosamine [37]. Lectin isolated from the fruiting bodies of orange peel fungus *Aleuria aurantia* is fucose specific [38]. The hemagglutinin of *Aspergillus niger* is specific for L-arabinose and D-xylose [39]. Mucin, fetuin and their asialo derivatives inhibit lectins from *Rhizoctonia crocorum* and *Athelia rolfsii* [40]. Some fungal lectins show specificity for the $A_1$, $A_2$ and B red blood cells [41].

Slime mold, *Dictyostelium discoideum* has a galactose /N-acetyl galactosamine specific lectin, discoidin I, which is expressed only at the aggregating stage. In its isolated form, discoidin I agglutinates the aggregating slime molds but not the vegetative cells [42].

Lectin from influenza virus has been well-studied [43]. It is made of two types of subunits, HA 1 and HA 2, having molecular mass 36,000 and 26,000 respectively, covalently linked by disulphide bonds. The lectins are located on the surface of viral membranes and are specific for Neu Ac $\alpha$ 2-6 Gal and Neu Ac $\alpha$ 2-3 Gal.

***Plant lectins:*** Lectins from dicotyledonous plants for example, from the families of Solanaceae, Leguminoceae, Euphorbiaceae and Cucurbitaceae, have been extensively studied. In monocotyledonous plants, the Gramineae, Liliaceae, Orchidaceae and Araceae families have been investigated [44,45]. In some families like Leguminoseae and Gramineae, lectins are present in many species while in others like Euphorbiaceae, they are found only in a few species. Usually a particular plant source contains lectin(s) belonging to a single sugar specificity group but in a number of cases, two or more lectins differing in their sugar specificity are found in the same plant (Table I). Amongst cultivated plants, varietal differences exist and have been investigated mainly in legumes and in particular in *Phaseolus vulgaris* lectins (PHA). Of the 92 cultivars investigated, 7 had blood group

specific agglutinins, 75 had nonspecific agglutinins, 7 agglutinated animal red cells and 3 were negative [46].

Table I: Lectins with different sugar specificities present in the same plant source.

| Source | Lectin | Sugar-specificity | Blood group specificity | Ref |
|---|---|---|---|---|
| *Ulex europeus* | I | L-fucose | O | [28] |
| | II | GlcNAc | _ | |
| *Vicia cracca* | I | GalNAc | A | [47] |
| | II | Man, Glc | _ | |

Plant lectins often occur as isolectins. In diploids, a single subunit species is present, giving rise to one molecular form of the lectin. In polyploids however the different polypeptide chains coded for by different genomes combine randomly with identical or non-identical partners forming both homomeric and heteromeric dimers. Subunits of the same lectin usually have the same sugar specificity. Isolectins may show varying proportions of the constituent subunits like in *Griffonia simplicifolia* I isolectins with subunits A and B [28]. *G. simplicifolia* I, $A_4$ has specificity for N-acetyl galactosamine and $B_4$ for galactose. In cereals, subunits of lectins from wheat, rye, barley can be exchanged [48].

In plants, lectins are generally localised in the embryo, in the cotyledon of mature seed but are rarely reported in the seed coat. However, plant lectins are not restricted to the seed and may occur in other parts of

the plant too like, the potato tuber lectin [49], the leaf and bulb lectin of garlic [50] and the bulb lectin from *Sternbergia lutea* [51].

Most plant lectins are glycoproteins, exceptions being wheat germ agglutinin (WGA), concanavalin A (Con A), peanut and barley lectin. The glycoproteins are of two types, (a) those containing mannose and N-acetyl glucosamine like soybean and limabean agglutinin and (b) those containing arabinose and galactose like plants of the Solanaceae family. Several lectins of the first type also contain fucose and xylose [52]. The carbohydrate moieties are not essential for biological activity [53,54].

Majority of plant lectins can be classified into seven families of evolutionary and to some extent structurally related proteins. A schematic overview of the occurrence, structure and specificity of the lectin families is given in Table II [55]. Most lectins contain metal ions and some require metal ions for activity (Table III) [28].

Plant lectins have been purified by conventional ion exchange chromatography as well as by affinity chromatography on insolubilized polysaccharides like guar gum and arabinogalactan [56,57]. Table IV lists some affinity adsorbents used for the purification of plant lectins [52].

Physico-chemical properties of plant lectins are summarised in Table V [28,58]. Generally, legume lectins possess one while cereal lectins (WGA) possess two sugar binding sites per subunit. But some heterotetrameric lectins like *Abrus* agglutinin [59] and *Momordica charantia* lectin [60] have two sugar-binding sites per tetramer.

Plant lectins have been crystallised both with and without sugar. Table VI gives a list of lectin-sugar complexes that have been analysed at the atomic level [61].

Plant lectins that have been cloned include the bark and seed lectins from *Sophora japonica* [62] and the leaf, root lectin from garlic [63]. cDNA clones encoding the bark and seed lectins from *Sophora japonica*

have been isolated, their sequence analysed and alignment of the deduced amino acid sequence of the different clones revealed striking similarites between the mannose/glucose binding and the GalNAc specific lectins [62]. cDNA cloning of the leaf and root lectins of garlic, *Allium sativum*, revealed that the deduced amino acid sequences of the two lectins are virtually identical with the root lectin having an extra sequence at its C terminus. Smeets et al. [63] suggest, virtually identical precursor polypeptides are differentially processed at their C terminus in the roots and leaves of garlic and the differential processing yields mature lectins with strongly different biological activity. cDNA encoding the broad bean lectin (*Phaseolus vulgaris*) has been cloned in *E. coli* and characterised [64]. Mature seeds of *Erythrina corallodendron* were used as a source of mRNA for the construction of an expression cDNA library in the vector λZAP. The deduced amino acid sequence was identical to the sequence of the first 244 amino acids of EcorL as determined at the protein level except at 7 positions [65]. *Dolichos biflorus* contains a lectin namely DB58, in its stem and leaves, that is closely related to the seed lectin. The DB58 cDNA represents an m-RNA encoding a polypeptide of $M_r$ 29,545. The sequence of the DB58 lectin represents 84% homology to the *D. biflorus* seed lectin at the amino acid level [66]. Isolation and characterisation of cDNA clones encoding *Artocarpus integrifolia* isolectins has been reported [67].

It has been observed that lectin structures are highly conserved during evolution. Sequence homology of leguminous lectins reveal that all of them are homologous, provided one introduces appropriate deletions (Fig I.2) [68].

Table II: Overview of plant lectin families

| Lectin family | Taxonomic distribution | Approx. no.of identifi–ed lectins | Protom -er size (kDa) | No. of proto-mers | Nominal specificity |
|---|---|---|---|---|---|
| **Amaranthin lectin family Chitin-binding lectins** | Amaranthace -ae | <10 | 30 | 2 | GalNAc |
| **ClassI chitinases** | Ubiquitous | >50 | 30 | 1 | $(GlcNAc)_n$ |
| Gramineae subgroup | Gramineae | >10 | 18or [10+8] | 2 | GlcNAc |
| Heveins | diverse | <5 | 5 | 1 | $(GlcNAc)_n$ |
| Phytolacca subgroup | Phytolacca sp. | <10 | 10,15, 25,30, 35 | 2 | $(GlcNAc)_n$ |
| Solanaceae subgroup | Solanaceae | <10 | 10 | 2 | $(GlcNAc)_n$ |
| Others | diverse | <5 | | 1 or 2 | $(GlcNAc)_n$ |
| **Cucurbitaceae phloem lectins Jacalin related lectins** | Cucurbitaceae | <10 | 25 | 2 | $(GlcNAc)_n$ |
| Moraceae subgroup | Moraceae | <10 | [14+2] | 4 | Gal/ GalNAc |
| Mannose-specific subgroup | Monocots and dicots | <10 | 16 | 2 or 4 | Mannose /Maltose |

| | | | | | |
|---|---|---|---|---|---|
| **Legume lectins** | Legumes | >100 | 30 or [(30-x) +x] | 2 or 4 | Man/Glc<br>Fucose<br>Gal/GalNAc<br>$(GlcNAc)_n$<br>Sialic acid<br>Complex |
| **Monocot mannose-binding lectins** | Liliales, Arales, Orchidaceae, Bromeliales | >100 | 12or 30 or [(30-x)+x] | 1,2,3 or 4 | Mannose |
| **Type 2 RIP** | Monocots and dicots | >20 | [30-s-s-35] | 1,2or 4 | Gal/GalNAc or Siaα2-6Gal/GalNAc |

Table III: Metal content and requirement of lectins

| Lectin | Metal Content* | | | Metal Requirement |
|---|---|---|---|---|
| | $Mn^{++}$ | $Ca^{++}$ | $Zn^{++}$ | |
| Concanavalin A | 4.0 | 4.0 | | $Mn^{++}$, $Ca^{++}$ |
| *Euonymus europeus* | | 8.0 | 0.7 | |
| *Griffonia simplicifolia* | 1.2 | 2.0 | | $Ca^{++}$ |
| Lentil | 0.6 | 3.8 | | $Mn^{++}$ |
| Lima bean | 1.0 | 4.0 | | $Mn^{++}$ |
| Pea | 1.0 | 2.5 | | $Ca^{++}$ |
| Wax bean | 0.24 | 6.2 | | $Mn^{++}$ |
| L-PHA | 0.3 | 2.5 | | $Mn^{++}$, $Ca^{++}$ |
| *Ulex europeus* I | 0.42 | 2.0 | 0.82 | $Mn^{++}$ |

*Expressed as atoms per mole.

Table IV: Affinity adsorbents used for plant lectin purification

| **Matrix** | **Ligand** | **Lectin purified** |
|---|---|---|
| Sephadex | | ConA, lentil |
| Sepharose | | *Abrus precatorius,* |
| | | *Ricinus communis* |
| Chitin | | Wheatgerm |
| Sepharose | Ovomucoid | Potato, *D. stramonium* |
| | Thyroglobulin | *Phaseolus vulgaris* |
| Sepharose | α-aminocaproyl-N-glycoside of | |
| | Galactose | Soybean |
| Epoxy-activated | N-acetylgalactosamine | Soybean |
| Sepharose | | |
| | L-Fucose | *Ulex europeus* |
| Divinylsulfone | Galactose | Peanut |
| activated Sepharose | | |
| | Mannose | *Viccia ervilia* |
| Aminoethyl- | Melibiose | *Maclura pomifera* |
| acrylamide | | |
| | Lactose | *Ricinus communis*, |
| | | peanut |
| | Maltose | Lentil, Con A |

Table V: Chemical and Biological properties of plant lectins

| Source | Mr (Da) | Sub units (no.) | carbohydr. content (sugar) | Mitogenicity | Human bld grp specifi-city | Sugar specificity | n * |
|---|---|---|---|---|---|---|---|
| Jack bean | 102,000 | 4 | nil | + | non-specific | α-Man/ Glc | 4 |
| Horse gram | 140,000 | 4 | 3.8%, Man GlcN | | A | α-GalNAc | |
| Soybean | 120,000 | 4 | 6%, Man GlcNAc | | non-specific | GalNAc | 4 |
| Lentil | 42000 | 4 | 2%, Glc GlcN | + | non-specific | α-Man/ Glc | 2 |
| Lotus tetragonolobus | I:120000 | 4 | 9.4%, GlcN Hexose | | O | α-L-Fuc | 4 |
| | II:58000 | 2 | 4.8%, GlcN Hexose | | O | α-L-Fuc | 2 |
| | III: 117000 | 4 | 9.2%,GlcN Hexose | | O | α-L-Fuc | 4 |
| Gardenpea | 53000 | 4 | 0.3% Glc | + | non-specific | Man/Glc | |

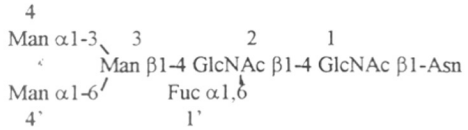
| Source | Mr (Da) | Sub units (no.) | carbohydr. content (sugar) | Mitogenicity | Human bld grp specificity | Sugar specificity | n* |
|---|---|---|---|---|---|---|---|
| *Ulex europeus* | I:65000 | 2 | 7.2%, Man,GlcN | | O | L-Fuc | |
| | II | | Man,Gal GlcN | | O | di N-acetyl chitobiose | |
| Castor bean | 120,000 | 4 | 12% | | non-specific | Gal | 2 |
| Potato | 100,000 | 2 | 50%, Ara | | non-specific | GlcNAc oligomer | |
| Wheat | 36,000 | 2 | 4.5% | | non-specific | GlcNAc & oligomers | 4 |
| Peanut | 110,000 | 4 | nil | + | | Gal | 4 |
| Tomato | 71,000 | | 50%, Ara Gal | | | β-GlcNAc oligomer | |
| Bitter gourd | 120,000 | 4 | | | non-specific | Gal | 2 |

* Number of binding sites per molecule

Table VI: Lectin carbohydrate complexes of known three-dimensional structure.

| Lectin | | Ligand | Resolution A° |
|---|---|---|---|
| Concanavalin A | Con A | Manα1-Me | 2.0 |
| Pea lectin | | Trisaccharide 3,4,4'[a] | 2.6 |
| *Lathyrus ochrus* lectin | LOLI | Manα1-Me and Glcα1-Me | 2.0/2.2 |
| | LOLI | Muramic acid and Muramyl dipeptide | 2.05 |
| *Griffonia simplicifolia* lectin 4 | GS4 | Lewis b tetrasaccharide[b] | 2.0 |
| *Erythrina corallodendron* lectin | EcorL | Gal β1-4 Glc | 2.0 |
| Soybean agglutinin | SBA | Pentasaccharide[c] | 2.6 |
| Wheat germ agglutinin | WGA | NeuNAc α 2,3 Gal β1-4 Glc | 2.2 |
| | | GlcNAc β 1,4 GlcNAc | 1.8 |
| Ricin | | Gal β 1-4 Glc | 2.5 |
| Snowdrop lectin | GNA | Man α1-Me | 2.3 |

Oligosaccharides designated by numbers refer to the residues indicated on the following oligosaccharide.




b) Fuc α1-2 Gal β1-3 GlcNAc
Fuc α1-4

c) Gal β1-4 GlcNAc β1-2
GalOH
Gal β1-4 GlcNac β1-2

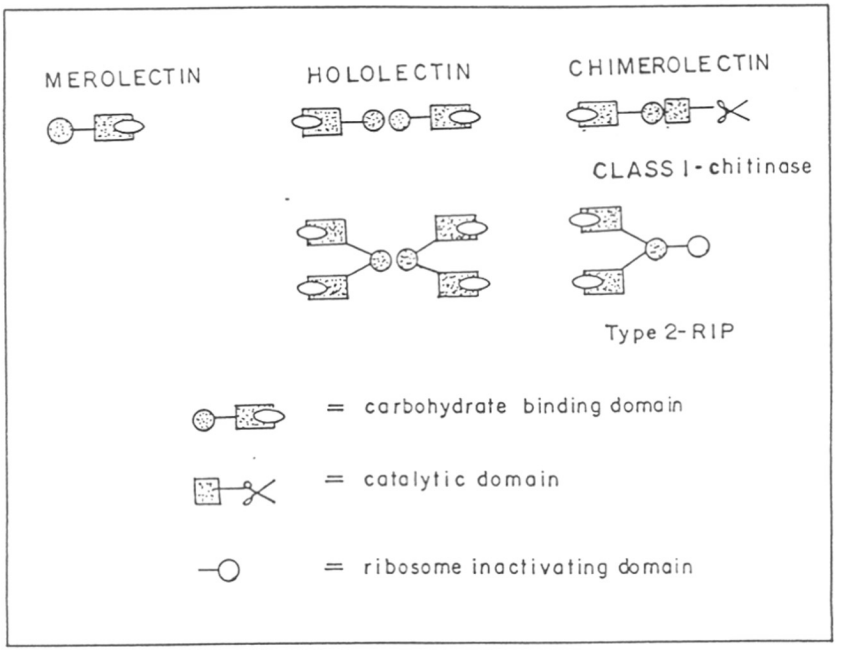



**Fig. I.1:**

**Schematic representation of three types of plant lectins:** merolectins, hololectins and chimerolectins.

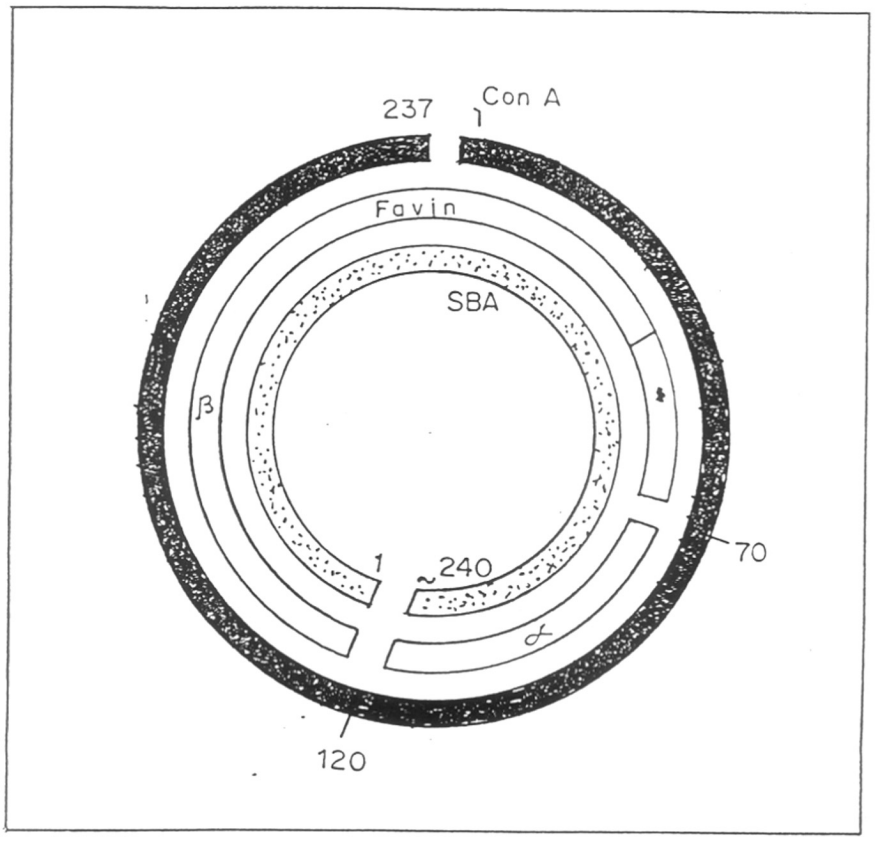



**Fig. I.2:**

**Circularly permuted sequence homology relating Con A with other leguminous lectins**: Fava bean lectin (favin) α and B chains (open bar), soybean agglutinin (SBA) (stipled bar) and concanavalin A (dark bar). The dashed line transecting favin β chain indicates the region where the homologous chain of the lentil lectin ends. The location of the covalently bound carbohydrate in favin is indicated by the asterick.

## CARBOHYDRATE SPECIFICITY

Lectins are characterised by their carbohydrate specificity. Determination of the specificity of lectins is important to evaluate their use as an analytical tool. Different methods for determination of association constants include the hapten inhibition technique, equilibrium dialysis and fluorescence spectroscopy [69,70].

Goldstein and Poretz [71] classified lectins into classes based on their monosaccharide specificity. Specificity of a lectin is often defined in terms of its monosaccharide inhibitor.

1) Mannose / glucose binding lectins –Leguminoseae .

2) N-acetyl glucosamine binding lectins (and its β 1-4 oligomers i.e. chitin oligosaccharides binding lectins) –Leguminoceae, Solanaceae and Gramineae.

3) Galactose and N-acetyl galactosamine binding lectins–Euphorbiaceae, Loranthaceae and Leguminoceae.

4) Fucose binding lectins – Occuring in seeds of *Lotus tetragonolobus* and *Ulex europeus*, the fungus *Aleuria aurantia* and in vertebrates like *Anguilla anguilla*.

5) Sialic acid binding lectins – Found in the haemolymph and in the sera of vertebrates and in plants like elderberry bark.

Most plants possess a single lectin but examples of plants having two or more lectins with different monosaccharide specificities exist, viz *Ulex europeus* has fucose and GlcNAc specific lectins [28]. Moreover, members of different lectin families may recognise the same sugar. Mannose binding lectins are found in legumes, monocots and in the family of jacalin related lectins. Galactose and N-acetyl–galactosamine specific lectins are found in the legume family, type 2 RIP and the amaranthins [55].

Some lectins bind di- and tri- saccharides more strongly than monosaccharides and their alkyl derivatives, indicating that the reducing sugar may be contributing to the binding energy of the lectin carbohydrate complex. Increased affinity for disaccharides as compared to monosaccharides is due to enthalpic effects, suggesting extended binding sites. Lectins also interact with polysaccharides and glycoproteins. The precise position of the determining sugar in a polysaccharide is important as most lectins interact with the terminal non-reducing glycosyl units of polysaccharides and glycoproteins [28]. However Con A, pea and lentil lectins interact with the terminal as well as internal mannopyranosyl residues [72,73]. The shape, size, valency and linkage of sugars influences specificity and affinity [74]. Isomaltose and other oligosaccharides having non-terminal α-D-1-6 glucosidic bonds have a higher affinity for Con A than oligosaccharides having non-reducing α-D-(1-2, -3, or -4) glucosidic linkage [75].

Some lectins do not have an efficient monosaccharide inhibitor and are inhibited by oligosaccharides, glycolipids, and glycoproteins; for example, *Agaricus bisporus* lectin is inhibited by glycopeptides from erythrocyte membranes, fetuin and IgA [76]. It is interesting to note that WGA specific for N-acetyl glucosamine oligosaccharides is also inhibited by the monomer [49] but *Datura stramonium* lectin inhibited by N-acetyl glucosamine oligomers is not inhibited by the monomer [76,45].

Glycosides with aromatic aglycons are often strong inhibitors because they interact with hydrophobic sites on the lectin. Lectins may also bind strongly to hydrophobic molecules, which do not contain saccharide structures [77]. When such binding is not inhibited by specific sugars, the non-polar site seems to be distinct from the carbohydrate-binding site.

Some lectins are specific for only one anomeric configuration. For example, Con A [75], *G. simplicifolia* [78] and *L. tetragonolobus* [79]

lectins are specific for the α-anomers of mannose (or glucose), galactose and L-fucose respectively. Some lectins like the soybean agglutinin [80] are devoid of anomeric specificity. Lectins from snake gourd [81] and *M. charantia* [82] prefer the β-anomer of galactose.

Sugars commonly employed in inhibition studies are in the pyranose form. For Con A, it has been found that fructose and arabinose in their furanose forms, are also inhibitors. Binding of furanosides to pyranose specific sites is of common occurrence in carbohydrate binding proteins [83].

Sugars that are good lectin inhibitors in solution do not always bind to immobilised lectins. With Con A [84] and *Ricinus communis* lectin [85] only carbohydrates with association constants in solution greater than $10^6$ $M^{-1}$ bind to immobilised lectins.

Proteins with more than one oligosaccharide sidechain interact with more than one lectin-binding site. These multivalent interactions, the cluster effect, gives rise to a higher affinity [61]. For some lectins, sugars linked to asparagine or serine/ threonine have a higher affinity than the sugar alone [86].

Kinetics of lectin-sugar interaction generally agrees with a one step bimolecular reaction. The association and dissociation kinetics of carbohydrate binding is slow. This allows time for topological reorganisation of the crosslinked receptor complexes in the viscous cell membrane and time for interaction of the different receptors with the lectin molecule [87]. The association rates are about $10^5$ times slower than that for a diffusion-controlled process [88-90].

Molecular shape is important in determining lectin sugar interactions. *Ulex europeus* lectin binds to both L- Fuc α 2 Gal β 4 GlcNAc and L-Fuc α 2 Gal β 3 $GlcNH_2$ but not to L-Fuc α 2 Gal β 3 GlcNAc. The

first two are structurally different but topographically similar whereas, the third trisaccharide is dramatically different topographically [91]. WGA is specific for oligosaccharides of N-acetyl glucosamine and it also binds to N-acetyl galactosamine and sialic acid. The binding of sialic acid and N-acetyl galactosamine to WGA is possible because of the configurational similarity of these sugars with N-acetyl glucosamine, at position C-2 (N-acetamido) and C-3 (hydroxyl group) of the pyranose ring. C-2 and C-3 are critical positions for WGA-sugar interaction [92].

In addition to the nature of the participating entities, the reaction medium (usually water) and the experimental conditions play an important role in making the interaction feasible [93]. The participation of hydrophobic bonds in carbohydrate binding to lectins has been acknowledged, therefore polarity reducing agents like ethylene glycol and mild denaturing agents added to the medium lead to the enhancement of lectin specificity.

***Lectins and their interaction with oligosaccharides and glycoproteins:*** High mannose type glycopeptides and certain bisected hybrid type N-linked glycopeptides form homogenous aggregates with tetrameric Con A [94-96]. The homogenous nature of the precipitate appears to be due to the unique cross-linked lattice of each complex. The specificity of binding to Con A in their cross-linked complexes is far greater than in the corresponding soluble complexes. Equimolar mixtures of two glycopeptides form a unique lattice with the lectin, which excludes the lattice of the other glycopeptide. The glycopeptide with lower affinity shows a precipitation maximum at a lower concentration than the one with higher concentration. Two protein binding sites are identified on each arm of the glycopeptide, one site on the $\alpha(1\text{-}6)$ arm of the core, which binds with high affinity (primary site) and the other on the $\alpha(1\text{-}3)$ arm of the core which binds with lower affinity (secondary site). Certain bisected hybrid type glycopeptides were found to possess only

the primary type sites and hence were able to bind but not precipitate the lectin. Since other related glycopeptides have only the secondary type sites they exhibit low affinity and are unable to precipitate the protein [97].

Studies with synthetic bisected and non-bisected complex type oligosaccharides and related glycopeptides show that certain oligosaccharides of the former group are bivalent and capable of binding and precipitating Con A. The corresponding non-bisected analogues bind but do not precipitate as they are univalent [98]. Dimeric derivatives of Con A (acetyl Con A and succinyl Con A) showed lower affinities than tetrameric Con A, towards certain oligomannose type glycopeptides [99].

Multiantennary complex type oligosaccharides and glycopeptides with non-reducing terminal galactose residues undergo bi-, tri- and tetra-valent interactions with a variety of galactose specific lectins, leading to precipitation of the complexes. The valency of a carbohydrate is generally equal to the number of chains with non-reducing terminal galactose residues, although the size of the lectin monomer also influences the valency of the oligosaccharide. The extent of precipitation is dependent not only on the valence of the oligosaccharides but on their branching patterns as well. A tetravalent lectin appears to be required to cross-link biantennary oligosaccharides [100]. Difucosyl biantennary oligosaccharides are capable of cross-linking and precipitating with tetrameric isolectins of *Lotus tetragonolobus*, LTL-A and LTL-C but not with dimeric isolectin LTL-B. The precipitates of LTL-A formed in the presence of a mixture of two oligosaccharides, show patterns characteristic of one or the other oligosaccharide, depending on the relative concentrations of the carbohydrates. Heterogenous complexes involving two different oligosaccharides bound to a lectin do not precipitate presumably due to the thermodynamic instability of such lattices [101]. Direct evidence from electron microscopy showed the existence of long range order in cross-

linked complexes between N-linked oligosaccharides and lectins from *Lotus tetragonolobus* isolectin A and soybean. The degree of organisation in the precipitates formed resembles a crystalline lattice [102].

Effect of the protein environment on the binding properties of oligosaccharides has been examined through interactions of Con A with soybean agglutinin (SBA). The total valency of the carbohydrate of SBA is a function of both the quaternary structure of Con A and the ratio of SBA to Con A. The individual Man 9 oligosaccharide, which as a glycopeptide is bivalent for binding to Con A, expresses univalency when present on the protein matrix of SBA [103].

Con A binds and forms unique cross-linked complexes with different glycoproteins having different numbers and types of carbohydrate chains as well as different quaternary structures. Heterogeneous complexes, involving two different carbohydrates cross-linked to a lectin, do not form presumably due to lack of long range order in such complexes. Stability in the homogeneous cross-linked complexes may also be due to similar close molecular packing interactions and long range order in the lattices [104].

Results with a 14 kDa S type, β-Gal specific animal lectin demonstrated that the lectin possesses similar but distinct cross-linking activities, compared to several galactose specific plant lectins, towards asialofetuin (ASF). Both plant and animal lectins are capable of forming highly ordered cross-linked lattices with specific glycoproteins. Unlike plant lectins, the 14 kDa lectin fails to precipitate with the free triantennary glycopeptide or oligosaccharide from asialofetuin or with other related branched chain carbohydrates, suggesting an important difference in the cross-linking activities of the animal lectin compared to plant lectins. The total valency of the carbohydrate chains of asialofetuin for a series of galactose specific plant lectins and an animal lectin is determined by (i) the number and composition of the carbohydrate chains on ASF (ii) the

quaternary structure and size of the lectins and (iii) relative ratio of the glycoprotein to lectin in solution [105].

There are two types of cross-linking interactions [106]. Type 1 is a complex formed between bivalent carbohydrate and a bivalent lectin. It is a linear (one-dimensional) soluble complex [99,100] (Fig. I.3a). Type 2 complexes are formed between carbohydrates and lectins in which one molecule possesses a valency greater than two. Two and three-dimensional complexes are formed which are generally insoluble [97,98,100]. The complex formation is reversible as non-covalent interactions are involved. Two and three-dimensional cross-linking may be homogeneous if crystal packing interactions are present, if not, heterogeneous cross-linked complexes may form. A homogeneous complex is one where one carbohydrate and one lectin exist in a symmetrical repeating unit. The lattice is ordered (Fig. I.3b). Heterogeneous cross-linked complexes involving two different carbohydrates bound to a lectin showed no evidence of precipitation.

Specificity of binding in a type 2 cross-linked complex is much greater than the corresponding solution complex and greater than the type 1 complex. Affinities of the lectin and carbohydrate for a homogenous 2/3 dimensional lattice is greater than the corresponding solution complex due to multivalent binding of each molecule to the lattice.

Each carbohydrate forms its own unique cross-linked lattice with the lectin even in the presence of mixtures of the molecules. The carbohydrate crosslinking patterns of lectins are a key feature of their biological activities. Cross-linking of glycoconjugates has been implicated in the mitogenic activities of lectins [107] and in the induction of mating reactions in fungi [108].

Most plant lectins with closely spaced domains form homogeneously cross-linked complexes with carbohydrates and glycoproteins possessing

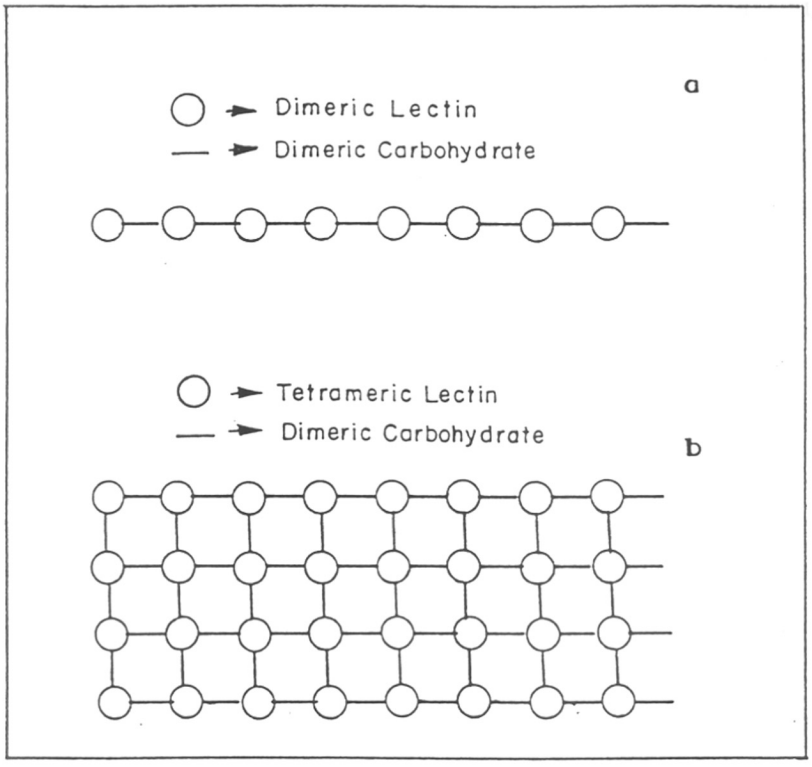



**Fig. I. 3:**

a) Schematic diagram of a type 1 cross-linked complex between a divalent lectin and a divalent carbohydrate.
b) Schematic diagram of a type 2 cross-linked complex between a tetravalent lectin and a divalent carbohydrate.
In both schemes lectin is represented by a circle (O) and the carbohydrate by a line (—).

single or tandemly arranged domains. C type $Ca^{++}$ dependant lectins like collectins possess multiple lectin domains seperated by large spacer domains (separated binding domains). These types of lectins form loose heterogeneous cross-linked networks with glycoconjugate receptors.

## STRUCTURAL BASIS OF CARBOHYDRATE SPECIFICITY

Quiocho and colleagues have categorised carbohydrate-binding proteins into two groups [109,110]. In group I proteins, the carbohydrate binding sites are located in deep clefts and binding leaves little or none of the ligand exposed to the bulk solvent. Binding affinities are very high as virtually all the hydrogen bonding potential of the sugar is used and favourable entropy gain is obtained from expelling large number of water molecules when the protein closes around the ligand [111]. The bacterial periplasmic binding proteins and enzymes that act on sugar substrates are best known examples of this group. In group II proteins, the binding site tends to be in shallow clefts, binding affinities are lower and proteins tend to form hydrogen bonds with only a subset of the OH's of the sugar. Lectins belong to this second group of proteins.

In lectins, "Nature seems able to construct saccharide binding sites on very different frameworks as exemplified by legume lectins with their large β structures and low cysteine content as well as WGA with its high cysteine content and virtual absence of regular structure on the other" [112]. The contact amino acids are not necessarily the major determinants of specificity. Specificity is determined primarily by the exact shape of the combining site (shape of residues lining the binding pocket, outline of the β turns) and the precise spatial disposition of the surrounding. Therefore, small changes in the site may result in major changes in specificity [113].

Lectins from leguminous plants constitute a large family of homologous proteins with remarkably different carbohydrate specificity. The differential specificity arises due to the variability in size and conformation of the binding site loops [114]. The overall 3D structure of legume lectins seems better conserved than the primary structure. The *Lathyrus ochrus* lectin I (LOL I) (Man, Glc specific) and *Erythrina corallodendron* lectin (ECorL) (Gal specific) are good examples as in each case, seven hydrogen bonds hold the monosaccharide in the combining site. The side-chains of two conserved residues, an aspartic acid and an asparagine, form 3 key hydrogen bonds. In EcorL, galactose is rotated in the combining site so that its 3-OH (equatorial) and 4-OH (axial) forms hydrogen bonds with Asp 89 and Asn 133 whereas, in LOL I, the mannose or glucose forms hydrogen bonds to the equivalent protein side chain atoms with its 4-OH (equatorial) and 6-OH i.e. the sugar is rotated so that a different set of OH's are involved in these interactions, the 4- and 6-OH's of mannose versus the 4- and 3- OH's of galactose. van der Waal's contacts occur predominantly with ring atoms of the aromatic residues that are coplanar with the pyranose ring of the ligand [109].

The cereal lectins such as WGA are structurally different than the legume lectins. Two independent non-cooperative binding sites are located at the interface between the subunits that form the wheat germ agglutinin dimer. All sialic acid ring constituents are involved, the acetamido group making the largest number of contacts with the lectin and only one water molecule appears to play an important role in stabilizing the lectin-sugar complex [113].

The C type animal lectins display different types of sugar selectivity as compared to plant lectins. Fundamental aspects of the sugar binding mechanism are shared in the mannose type and galactose type binding sites. In particular, the 3- and 4- OHs of both types of sugars, although differently

disposed, are apical coordination ligands for a bound $Ca^{++}$. Discrimination results from steric exclusion and aromatic packing interactions, which are unique to galactose [115] and interchange of the positions of single amide and carboxylate oxygen. C type lectins, which recognize mannose, contain glutamate and asparagine and those that recognize galactose have glutamine and aspartate at the corresponding position. This suggests that the side-chain arrangement at the two positions may be a primary determinant of specificity of the C type lectins [116].

Specificites and affinities of the binding sites of lectins are achieved through hydrogen bonds, van der Waal's contacts and hydrophobic interactions [117,118].

*Hydrogen bonding* - Cooperative hydrogen bonding, in which the hydroxyl group OH acts simultaneously as a H bond donor and acceptor, is characteristic of the interaction of lectin with sugar hydroxyls. Generally, acidic chain, carbonyl or carboxylate is used as a H bond acceptor from sugar hydroxyl while donors come from the main chain amide groups. Charged side chain donors also occur frequently, but there is infrequent use of protein OHs from tyrosine, serine, threonine as either donors or acceptors of hydrogen bonds with sugar OHs [61]. Specificity is derived from hydrogen bond geometry specific to a single epimer. Concanavalin A and pea lectin, both of which bind to mannose and glucose, form specific hydrogen bonds with 3-, 4- and 6-OHs of the sugars but not the 2-OH, which differs between these two monosaccharides [119]. Snowdrop lectin (GNA) specific for mannose, forms hydrogen bonds with 2-OH in addition to other OHs [120].

Comparison of the binding sites in liganded and unliganded states, shows that some water molecules bound to the protein in the sugar free state remain in the presence of sugar and form hydrogen bonds with the ligand. In this case, water molecules act as fixed structural elements

equivalent to hydrogen bonding groups of the protein and can therefore be considered as a part of the binding site architecture [121].

In the recognition of sialic acid by lectins like WGA [122] and influenza hemagglutinin [123], the carboxylate moiety interacts with the main chain amide groups, polar side chains (especially serine) and ordered water molecules rather than fully charged side chains in hydrogen bond interactions.

Sugars have significant non-polar patches, which is frequently packed against the face of one or more aromatic side chains. The axial disposition of 4-OH of galactose as opposed to the equatorial 4-OH of mannose creates a more extensive and continuous non-polar surface. Therefore, galactose has been found packed against an aromatic side chain but the situation with mannose is variable. The carbon backbone of the glycerol moiety of N-acetyl neuraminic acid also presents an apolar surface and packs against the face of tyrosine in WGA [122].

Legume lectins use $Ca^{++}$ and $Mn^{++}$ to stabilise the binding site and fix the positions of amino acids that interact with sugar ligands. The $Ca^{++}$ coordination shell fixes side chains for optimal binding to the sugar. $Mn^{++}$ fixes the $Ca^{++}$ position but does not coordinate any residue that directly interacts with the protein. A direct role for cations is observed in C type lectins where $Ca^{++}$ forms direct coordination bonds with the sugar ligand [124]. The coordinate bonds, which link sugars to $Ca^{++}$ contribute substantially to the affinity of this binding site. These are the only known lectins that exploit this energetically favorable interaction.

Extended sites or subsite multivalency describes binding sites that interact specifically with more than one sugar residue to provide increased affinity [125]. Substantial affinity enhancements are observed when additional sugars are linked to the primary determinant even though the lectin does not show significant affinity for the second sugar as a

monosaccharide. Some lectins do not show affinity for a monosaccharide but specifically bind to larger oligosaccharides. For example, galectins have a marginal affinity for galactose but bind lactose and N-acetyl lactosamine with high affinity [126]. In the extended binding sites, water molecules play a dual role. They stabilise the conformation of the oligosaccharide by forming hydrogen bonds among the OHs and ring oxygens of different sugars. Secondly, they mediate interactions between protein and ligand.

Lectins can have two clearly separated and thermodynamically independent binding sites in a single protomer as in WGA [127] and GNA [128]. In no case there is evidence of molecular cooperativity and the lack of cooperativity is consistent with the conclusion that binding sites are preformed and proteins change little upon sugar binding. In some cases, secondary sites with no sequence or structural homology to the primary site have been identified. In these cases, the secondary sites appear to have much lower affinity for the ligand than the primary site but their significance is unclear [124].

The binding sites of lectins are preformed with water molecules forming hydrogen bonds with unliganded protein, in a pattern that closely mimics hydrogen bonding by sugar OHs [119]. There is no evidence of distortion of the sugar ring geometry on binding to a lectin, in contrast to hydrolytic enzymes that cleave the glycosidic bond.

The increase in affinity for oligosaccharides can be a result of extended binding sites or can result from clustering of several similar binding domains through formation of polypeptide oligomers. Ideally, the net free energy of binding can be the sum of the free energies of the individual binding interactions. In reality, substantial increase in affinity is seen but not true additivity. In cluster ligands, spacing is critical for optimal binding. Spacing of binding sites on a multivalent lectin must match a

preferred oligosaccharide conformation, else it becomes energetically costly for the rearrangement of the ligand to fit the lectin [128].

Thus, the ability to bind carbohydrates has evolved independently as evident from the lack of similarity among different groups of lectins. Inspite of that, a few common features of the binding sites can be listed. Sugar binding sites are a shallow indentations on the protein surface and these are mostly preformed. Charged or polar planar groups form majority of the hydrogen bond donors and acceptors with the sugar OHs. Selectivity is achieved through a combination of hydrogen bonding to the sugar hydroxyl groups with van der Waal's packing and often hydrophobic stacking interactions. Higher selectivity is achieved by extending binding sites through additional direct and water mediated contacts between oligosaccharides and the protein surface. Dramatically increased affinity for oligosaccharides results from clustering of simple binding sites in oligomers of the lectin polypeptides. Differential sugar recognition is achieved by involving (i) the epimeric OH that distinguishes sugars (4-OH in mannose and galactose) in hydrogen bonding to the lectin, (ii) by steric exclusion of certain ligands and (iii) through accommodation of multiple ligands by projecting away those portions of the ligands that would allow them to be distinguished [61].

## ROLE OF LECTINS

Cells have the ability to recognize and respond to one another. The exact chemical basis for this is not fully understood. Certainly, proteins play a part in recognition but evidence suggests that carbohydrates are the primary markers for cell recognition. Earlier, carbohydrates were considered only as structural materials and energy sources. Subsequently it was realised that they have an enormous potential for encoding biological information-the number of monomers, their sequence, linkages, branch

points and anomeric configuration being important. Hence, while 4 different amino acids or nucleotides can form only 24 tetrameric structures, 4 different monosaccharides can form 35,560 distinct tetrasaccharides [129]. Thus, sugars are information-bearing molecules with a vast potential for structural diversity and as lectins can distinguish between different monosaccharides and even detect subtle changes in oligosaccharides, they have been thought to be the recognition molecules in nature.

**Animal lectins**

*Killing of microbes*: Macrophage membrane lectins can function in lectinophagocytosis. Mannose and N-acetyl glucosamine specific lectins of guinea pig alveolar macrophage can mediate the binding and lectinophagocytosis of microbes like *Aspergillus fumigatus* [130].

*Recognition of tumor cells* - Galactose and N-acetyl galactosamine specific lectins of elicited mouse peritoneal macrophages appear to be involved in distinguishing tumor cells from normal ones and in killing the tumor targets [131]. Staining with antibody to the purified lectin revealed that the lectin is present on the surface of the activated macrophages but absent from resident macrophages.

*Cell differentiation and organ formation*: A β-galactoside specific lectin found in the epithelium of the thymus is postulated to be responsible for holding immature thymocytes in the thymic cortex by binding to galactose residues on the surface of these cells [132]. On maturation of the thymocytes, the galactose residues become masked by the attachment of sialic acid, the cells lose their ability to bind the lectin and they are thus free to migrate to the thymic medulla where the mature thymocytes reside or to directly enter the circulatory system.

*Migration of lymphocytes:* During their normal life span, lymphocytes migrate from the blood stream into lymphoid organs like Peyers patches and lymph nodes. Different lymphocytes home towards different organs.

An adhesive interaction between lymphocytes and the endothelium of post capillary venules is the first step in this migratory or homing process and the recognition is based on lectin-sugar interaction [133].

*Metastasis:* Galactose specific lectins present on various human and murine tumors are suggested to influence the pathogenesis of cancer metastasis by promoting the formation of tumor cell aggregates (emboli) in the circulation and their adhesion to the endothelial layer of capillaries [134].

*Host defense*: Galactose specific lectin from *Viscum album* binds to a variety of hemopoietic cells, induces increase in clonogenic growth of haematopoietic progenitor cells [135] and thus increases cellular parameters of defense.

**Protozoan lectins:** Once amoebae have invaded the host, lectins mediate the binding of the parasite to cells especially hepatocytes, initiating the killing of these cells. In addition, the lectin enables amoebae to bind bacteria carrying the appropriate sugars. The bound bacteria subsequently serve as a source of nutrition for the parasites [136]. In *Paramecium primaurelia*, lectin-binding sites are involved in mating pair formation [137].

**Bacterial lectins:** Bacterial adhesion varies between tissues and between species. It is mediated by surface lectins of the bacteria, which bind to complementary sugars on the host cells. Adhesion to the target tissue is critical for bacterial infection. Different bacteria may bind to different parts of the same carbohydrate. Inorder to interfere with bacterial adhesion any agent that competes with the bacterial lectin can be used, for instance antibodies against mannose for mannose specific *E.coli* [138]. In phytopathogenic bacteria, the fimbrial lectin is responsible for the attachment to host plant cell surface, attachment being the first step in infection [139]. Lectin carrying bacteria may also bind to sugars on

phagocytes resulting in phagocytic activation, ingestion of bacteria and their death [140].

**Lectins from slime molds:** An endogenous lectin discoidin I, synthesized as the mold aggregates, functions to promote cell substratum attachment and ordered cell migration during morphogenesis rather than cell adhesion as earlier proposed [141]. Two mutants of *D. discoideum* with low levels of discoidin I failed to form streams and to spread on plastic but were only partially impaired in their ability to form aggregates.

**Fungal lectins:** Some believe that fungal lectins are storage proteins [142]. Others propose their possible involvement in morphogenesis and development [143]. Fungal lectins appear to be involved in the recognition between lichen symbionts [144], interactions with insects [145] and plants [146]. In yeasts, flocculation and fusion of haploid cells of opposite mating types is believed to be mediated by agglutinins [147].

**Viral lectins:** Lectins play a role in viral infection. Binding of influenza virus hemagglutinin to sialic acid containing carbohydrates, on the surface of target cells, leads to the fusion of the viral and cellular membranes which facilitates the release of the viral genome into the cytoplasm and its subsequent replication. Removal of sialic acid from the cell membrane abolishes the binding and prevents infection whereas, insertion of sialic acid containing oligosaccharides restores the binding ability of the cells thus confirming the role of lectins in infection [148].

**Plant lectins**

*Mediators of symbiosis:* It was suggested that rhizobial attachment to plant root occurs by direct interaction between bacterial surface carbohydrates and lectins present in the roots. However, the lectins used in invitro studies were isolated from the seeds and not the roots. "Trifolin" of white clover is an example of a lectin isolated from the roots that can bind to the specific nodulating strain of *Rhizobium* [149]. The lectin has been suggested to

reversibly crossbridge receptors on the root hair cell wall with bacterial capsular polysaccharides and/or lipopolysaccharides as a prelude to nodulation.

*Pisum sativum* lectin (PSL) is abundant in the seeds and present only in small amounts in the roots. The root PSL and seed PSL are products of the same 'psl' gene [150]. When the 'psl' gene was introduced into hairy roots of white and red clover, pea rhizobia appeared to introduce nodule like structures and few nodules in the transgenic roots in contrast to the control roots [151]. Kijne et al. [152] proposed that lectins released by the roots of legumes may have a role in nodule developement.

*As nutrition source:* Developemental studies indicate that lectins are deposited in seed cotyledons during later stages of developement and are degraded to varying degrees following germination [153].

*Anchor*: Lectins in the phloem exudate of cucurbits [154] anchor filamentous proteins in the sieve tube, to sieve tube glycoproteins and provide strength to the sieve tube [155].

*Protection against phytopathogens:* A major argument used in favour of a defensive role for plant lectins is the observation that some plant lectins bind glycoconjugates not found in plants but which are present in other organisms. Sialic acid binding lectins from elderberry bark (*Sambucus* species) [156] bind sialic acid, which is absent in plants but forms a major constituent of animal glycoproteins. Chitin binding plant lectins recognize a carbohydrate that is a typical constituent of the exoskeleton of invertebrates but not found in plants. Moreover, most plant lectins show a marked stability under unfavorable conditions and in this respect resemble other defense-related proteins like protease and α-amylase inhibitors. The preferential association of lectins with those parts of the plant that are most susceptible to attack by foreign organisms is also an argument in favour of their protective role [11].

The lectin from winter aconite (*Eranthis hyemalis*) [157] is very toxic to the larvae of *Diabrotica undecimpunctata,* a major insect pest of maize. The loss of motility of bacteria at the air water interface of thornapple, *Datura stramonium* could be ascribed to the seed lectin and the effects are fully reversed by fetuin which is firmly bound by the lectin [158]. Hevein from the latex of the rubber tree has an anti-fungal activity comparable to that of nettle lectin [12]. The only plant lectins that can be considered fungicidal are the chimerolectins belonging to class I chitinases and their fungicidal properties reside in their catalytic domain rather than in their carbohydrate-binding domain.

Epithelial cells along the digestive tract of insects are directly exposed to contents of the diet and since glycoproteins are major constituents of these membranes, the gut is covered with potential binding sites for dietary lectins. Lectins from wheat germ had an inhibitory effect on the development of larvae of cowpea weevil [159] and Snowdrop lectin showed a high toxicity towards sucking insects, in experiments with transgenic plants [160]. Arcelin-I is another insecticidal lectin like glycoprotein from *Phaseolus vulgaris* L [161].

Similarly, the digestive tract of animals offers countless targets for interactions with dietary plant lectins. Ingestion of PHA or raw beans causes acute nausea and vomiting as the lectin induces enhanced metabolic activity that eventually leads to hyperplasia and hypertropy of the small intestine [162]. Lectins of black locust and elderberry also cause severe toxicity which could possibly be why elderberry and black locust are never attacked by rodents, deer or other wild life whereas barks of the surrounding lectin free species like poplar, willow, wild apple are a favorite food for the same animals [11]. Although the presence of toxic lectins may not completely protect the seed or plant from consumption, the reaction of avoidance by the animal might prove beneficial for the survival of the

species. Peumans and Van Damme therefore put forth a strong case for the defensive role of plant lectins in nature [11].

Despite their abundance, plant lectins are enigmatic in their true physiological functions in contrast to mammalian lectins. The physiological role of lectins as recognition molecules in animals is by virtue of their exquisite specificity, speed of interaction and reversibility. In reality, recognition may require lectins having distinct specificites along with other classes of molecule such as integrins and antibodies [148].

## APPLICATIONS

Lectins find wide application in biology, primarily for structural studies, purification purposes and for detecting cell surface changes.

***Isolation/fractionation of glycoproteins and glycopeptides:*** Covalently coupled lectins have been used for the affinity purification of a large variety of biological molecules such as hormones, enzymes, immunoglobulins and blood group substances. The purification of membrane glycoproteins is commonly carried out in the presence of non-ionic detergents like Triton X-100 and Nonidet–40 on immobilised lectins (Table VII). Insulin [163] and epidermal growth factor receptor [164] are some other glycoproteins purified by affinity chromatography on immobilised lectins.

Affinity chromatography on lectins is also useful for the fractionation of glycoproteins and glycopeptides, which differ slightly in their carbohydrate composition or in the structure of their oligosaccharide units. Concanavalin A is able to resolve mixtures of closely related glycopeptides [169](Fig. I.4). Triantennary glycopeptides of the type A, are not at all adsorbed on Con A-Sepharose, type B are slightly retarded, those of type C are eluted with low concentrations of methyl α-glucoside and type D are only eluted with high concentrations of sugar.

Table VII: Purification of membrane glycoproteins

| Material purified | Source of membrane | Immobilised lectin | Ref |
|---|---|---|---|
| Viral glycoprotein | Enveloped viruses(influenza) | Lentil | 165 |
| Rhodopsin | Bovine retina | Concanavalin A | 166 |
| Glycophorin | Human erythrocytes | Wheat germ | 167 |
| Acetylcholine receptor | Rat brain | *Lotus tetragonolobus*, *Ricinus communis* | 168 |

**Fig.I.4: Differential Affinity of Con A for oligosaccharide structure**

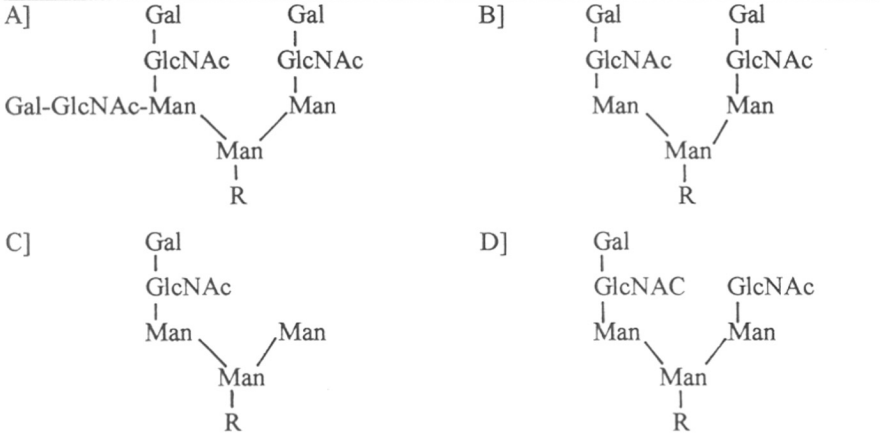
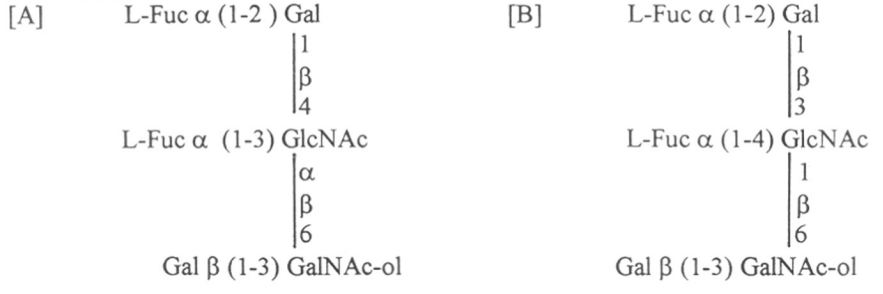



**Fig. I.5: Blood group Hexasaccharide**



***Isolation of glycosylated nucleic acids:*** t-RNA$^{asp}$ from rat and rabbit liver was isolated on a column of Con A-Sepharose while t-RNA$^{tyr}$ from the same sources was purified on a column of *R. communis* agglutinin–Sepharose [170].

***Structural studies on carbohydrates:*** Methylation analysis of the blood group hexasaccharide can only show that there is a 1-3 and a 1-4-substituted N-acetyl glucosamine residue but cannot establish which of the two is 1-3 linked or 1-4 linked. The structure can be unambiguously established with the help of *Lotus tetragonolobus* lectin, as structure A is bound by the Lotus lectin while structure B is not [171] (Fig. I.5).

***Characterization of cell surfaces:*** In addition to redistribution on the cell surface, lectin receptors can rotate in the plane of the membrane [172-174]. These observations provide evidence for the fluid mosaic model for membranes.

Receptors on the lymphocyte are evenly distributed over the surface, immediately after lectin binding. Within minutes, at 37°C (but not at 4°C), the receptors move in the plane of the membrane to form clusters or patches. On a fraction of the lymphocytes (10-40 %) the patches aggregate to form a cap at one pole of the cell [172]. Patching appears to be a simple diffusion process while capping requires energy. The two processes induced by lectins are similar to those caused by anti-immunoglobulins and other antibodies to lymphocyte surface constituents [175].

***Cell separation:*** Two approaches are used to separate intact cells bearing different surface carbohydrates. Cells may be separated by differential agglutination with soluble lectins or by affinity chromatography on insolubilized lectins. Leukocytes were separated from erythrocytes by agglutination with PHA [176]. Mouse thymocytes can be separated into mature, medullary and immature, cortical subpopulations using peanut agglutinin [177]. The agglutinated immature cells were separated from the

unagglutinated mature ones by sedimentation at unit gravity and dissociated into single viable cells by galactose. Mouse spleen T and B cells can be separated by selective agglutination of the latter with soybean agglutinin [178]. HeLa cells bound to immobilized lentil lectin could be removed from the matrix in high yield and in viable form on addition of specific sugars [179].

***Clinical uses:*** Mitogenic lectins are used to recognize congenital and acquired immunological diseases and deficiencies as well as to monitor effects of immunotherapeutic manipulation [180].

In blood banks, lectins are used to detect secretors, to identify $A_1$ and $A_2$ subtypes of the A blood group, to distinguish between M and N cells and to diagnose polyagglutination (agglutination of human red cells by antibodies in the sera) which may happen due to the appearance of T antigen on the erythrocytes by the action of neuraminidase of the infective bacteria or virus.

In animal experiments [181], liver lectin blockade with galactose containing receptor analogues has been shown to inhibit metastatic spread into the liver.

***Histochemical and cytochemical studies:*** Changes in lectin binding pattern have been observed during embryonic differentiation [182] cell maturation [183] and malignant transformation [184]. *Artocarpus integrifolia* lectin binding specifically to the T-antigen (Gal β1-3 GalNAc), a tumor associated antigen, can be used as an anti T probe in cancer research [185]. *Ulex europeus* lectin I binds specifically to vascular endothelium [186] and facilitates the detection of vascular invasion by tumor cells.

***Mapping of neuronal pathways:*** Lectins conjugated to horseradish peroxidase have proved useful in mapping central neuronal pathways, since the conjugates are taken up by neurons and transported within the axons [187]. L-PHA [188] and ricin [189] conjugates are transported in the

anteretrograde and retrograde directions, respectively. Uptake of the lectin conjugate is apparently receptor (sugar) mediated.

***Typing of bacteria:*** Lectins can discriminate between the pathogenic and non-pathogenic strains of *Trypanosoma cruzi* and between different morphological stages of *Leishmania donovani* [190].

***Identification of cell surface carbohydrate:*** Steinmann and Stryer [191] have shown that the carbohydrate moiety in rhodopsin is accessible to Con A, suggesting that the carbohydrates are located on the surface of the disc membranes. Radiolabelled peanut agglutinin and *R. communis* agglutinin have been used to detect surface glycoproteins of human skin fibroblasts in polyacrylamide gels. Saccharide residues in normal human skin are localised by fluorescently labeled lectins [192].

***Cellular and subcellular architecture:*** Biochemical modification of sperm surface during maturation has been shown using lectins [191]. Distribution of lectin receptor sites on embryonic cells has been used to correlate their migratory behaviour and differentiation [193,194]. Differences in neonatal, embryonic and neoplastic mouse tissue have been demonstrated using lectins [195].

## OBJECTIVES OF THE PRESENT INVESTIGATION

Plant lectins and their biological properties have been a subject of investigation for a number of years. Lectins represent a diversified group of proteins differing in size, structure and composition. Based upon their sequence homology and structural features, lectins have been grouped into a few distinct families. Seed lectins from cereals and from the legume family have been widely studied. The genus *Artocarpus* belongs to the Moraceae family. Although occurrence of lectins has been reported in few *Artocarpus* species, lectins from only two species namely the *A. integrifolia* and *A. lakoocha* lectins have been characterised in detail. Both these lectins are inhibited by the T antigen disaccharide, which makes them tumor specific and useful in cancer research. Screening of different plant seeds, showed that the seeds of *Artocarpus hirsuta* contain a lectin with exceptionally high hemagglutination titre. Hence the present investigation was carried out to purify and characterise the *A. hirsuta* lectin, to understand its structure-function relationship and potential application.

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# CHAPTER II

# PURIFICATION AND CHARACTERISATION OF *ARTOCARPUS HIRSUTA* LECTIN

# SUMMARY

*Artocarpus hirsuta* lectin was purified to homogeneity by ammonium sulphate precipitation, negative adsorption on DEAE-cellulose followed by CM-Sephadex chromatography. It was also purified by affinity chromatography on guar gum. The purified lectin is a heterotetramer of molecular mass 60,000, consists of two pairs of non-identical subunits of Mr 15,800 and 14,200 and has a pI of 8.5. It is a glycoprotein and contains 6.25% neutral sugar. It agglutinates rabbit and human erythrocytes, does not require metal ions for activity. The lectin loses total activity at 60°C in 30 minutes.

Hemagglutination inhibition studies indicated that, among the monosaccharides, galactose is a better inhibitor of *A. hirsuta* lectin than glucose, mannose and fucose. The inability of glucose and mannose to inhibit the lectin activity can be correlated to the inversion of the C-4 hydroxyl group. The non-inhibitory nature of fucose indicates a possible role for the hydroxyl group at C-6. The hydroxyl group at C-2 has little influence on sugar binding to the lectin, as affinities for galactose, galactosamine and 2-deoxy galactose are similar. Methyl α-galactose was found to be the most potent inhibitor of the lectin and the T-antigenic disaccharide failed to bring about any inhibition.

The fluorescence of methylumbelliferyl α-galactose was totally quenched on binding to *A. hirsuta* lectin with no change in the emission maxima, indicating a change in the environment of the umbelliferyl moiety of the sugar occurred upon binding. This quenching was reversed on addition of methyl α-galactose. Scatchard analysis of the quenching data showed the presence of two sugar binding sites per molecule of *A. hirsuta* lectin.

# INTRODUCTION

The genus *Artocarpus* constitutes a group of plants whose fruits and timber are useful. Lectins have been isolated from the seeds of *Artocarpus* species. The first lectin to be isolated was from *A. integrifolia* [1]. Subsequently lectins were isolated from other *Artocarpus* species like *A. lakoocha, A. tonkinensis, A. champeden, A. incisa* and *A. altilis* [2-6]. Among these lectins, those from *A. integrifolia* and *A. lakoocha* have been well characterised.

The *Artocarpus* lectins have been purified on affinity matrices like IgA-Sepharose [7], Melibiose-agarose [8] and Affigel galactosamine agarose [9]. The *Artocarpus* lectins are $\alpha_2\beta_2$ type tetramers with molecular mass ranging between 39-70 kDa [5,8,10] and pI ranging between 5-9 [5,11,12]. They are glycoproteins, containing metals and showing wide pH stability. *A. lakoocha* and *A. integrifolia* show specificity for α-linked galactosides and are strongly inhibited by the T antigen disaccharide Gal β1-3 GalNAc [2,13]. However, the structure-function relationship of these lectins has not been studied in detail.

*Artocarpus hirsuta* is a member of the Moraceae family. The *A. hirsuta* tree is evergreen and grows southwards of the Konkan region of India. Its fruit is ovoid (2-3 inches long) and petioles as well as peduncles of this plant are hirsute with long tawny hairs [14,15]. The *Artocarpus* seeds contain very little fat, 77-86 % carbohydrate and 7-12 % protein [5,12]. The present chapter describes the purification and characterisation of a lectin from the seeds of *A. hirsuta.*

# MATERIALS AND METHODS

## *Materials*

### Chemicals

DEAE-Cellulose, CM-Sephadex C-50, guar gum, epichlorohydrin, acrylamide, N, N'-methylene bisacrylamide, sodium dodecyl sulphate, SDS-PAGE and gel filtration molecular weight markers, ampholines of pH range 3-10, Coomassie Brilliant Blue R-250, G-250 and the sugars used in the present study including 4-methyl umbelliferyl α-galactopyranoside, methyl α-galactose, methyl β-galactose, galactose, galactosamine, N-acetyl-galactosamine, 2-deoxygalactose, pNP α-galactose, pNP α-N-acetyl-galactosamine, pNP β-galactose, pNP β-N-acetyl-galactosamine, glucose, mannose, methyl α-glucose, methyl α-mannose, N-acetyl-glucosamine, N-acetyl-mannosamine, mannosamine, glucosamine, stachyose, lactose, Gal β1-3 GalNAc, rhamnose, xylose, raffinose and fucose were purchased from Sigma Chemical Company (St.Louis, USA). Sephadex G-100 was purchased from Pharmacia, (Uppasala, Sweden). All other chemicals used were of analytical grade. Microtitre plates were purchased from Tarson, (Calcutta, India).

### Seeds

The seeds were collected from Kerala, India, at the beginning of the rainy season. Their outer shells were rinsed with 70 % alcohol, the shelled seeds were air-dried and preserved at room temperature.

## *Methods*

### Protein determination

Protein concentration was determined as described by Lowry et al. [16] using bovine serum albumin as a standard.

### Hemagglutination and carbohydrate inhibition assays

Rabbit erythrocytes were washed thoroughly with 10 mM Tris-HCl buffer pH 7.2, containing 150 mM NaCl (THS) and prepared as a 3 % suspension in the same medium. Hemagglutination tests were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocyte suspension was mixed with 50 µl of serially diluted lectin and agglutination was examined visually after incubation for one hour at room temperature. The unit of hemagglutination activity (U) was expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the units of the lectin per mg of protein (U/mg).

Hemagglutination inhibition tests were performed similarly, except that serial dilutions of the sugar solution (25 µl) were pre-incubated for 15 minutes at room temperature with 25 µl of the lectin (minimum concentration showing titre 4). 50 µl of rabbit erythrocyte suspension was added, mixed and the plates read after one hour. The initial concentration of all the sugars used was 500 mM while that of paranitrophenyl sugars was 10 mM and that of Gal β1-3 GalNAc was 120 mM.

### Preparation of guar gum matrix

Guar gum was insolubilised by crosslinking with epichlorohydrin, in alkaline medium, according to Appukuttan et al. [17]. 50 g of guar gum powder was added with continuous stirring to the emulsion of 150 ml

sodium hydroxide (3 N) and 15 ml epichlorohydrin, till it solidified. The solidified mixture was first kept at 40°C for 24 h and then at 70°C for 12 h. The gel was soaked in water and homogenised in a blender.

**A] Purification of *A. hirsuta* lectin**

**Seed extract preparation:** 100 g of *A. hirsuta* seeds were shelled, crushed and soaked in THS. The suspension was stirred for 16 h at 4°C and filtered through a muslin cloth. The filtrate was centrifuged (10,000 rpm, 20 min) and the precipitate discarded. The clear supernatant was used in subsequent steps.

**Salting out:** The above supernatant was precipitated at 90 % ammonium sulphate saturation, by addition of solid ammonium sulphate under constant stirring at 4°C and allowed to stand overnight. The precipitate was collected by centrifugation (10,000 rpm, 20 min) dissolved in 20 mM Tris-HCl buffer pH 7.2 and extensively dialyzed against the same. The dialysate was centrifuged (10,000 rpm, 20 min) and the clear supernatant subjected to ion exchange chromatography.

**DEAE-cellulose chromatography:** The clear supernatant was loaded on a DEAE-cellulose column (4 cm × 20 cm) pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.2. The column was washed with the same buffer and the washings collected as fractions of 2ml until $A_{280}$ was below 0.02. Fractions showing absorbance greater than 0.2 and hemagglutination activity were pooled, concentrated and dialysed against 20 mM citrate phosphate buffer (CPB) pH 5.0.

**CM-Sephadex chromatography:** The above concentrate was adsorbed onto a CM-Sephadex column (4 cm × 20 cm) pre-equilibrated with the same buffer. The column was washed sequentially with CPB pH 5.0, CPB pH 6.0, phosphate buffer pH 7.2 and finally the bound protein was eluted

with phosphate buffer pH 7.2 containing 0.5 M NaCl. Fractions showing absorbance above 0.2 and hemagglutinating activity were pooled, concentrated by lyophilisation, dialysed against THS and stored at 0°C.

**B] Affinity Purification of *A. hirsuta* lectin**

The DEAE unadsorbed protein was pooled, concentrated and dialysed against THS. The dialysed protein was loaded on a guar gum column (2 cm × 25 cm) pre-equilibrated with the same buffer. After washing off the unadsorbed protein, the bound lectin was eluted with 0.2 M galactose in THS. Fractions of 2 ml were collected at the rate of 30 ml /h. Active fractions were pooled, dialysed against THS and stored at 4°C.

**Electrophoresis**

Purity was determined on a 7.5 % (w/v) analytical polyacrylamide gel at pH 4.5 [18] and the protein bands were visualised by Coomassie Brilliant Blue G-250 as well as by silver staining.

**Determination of molecular mass**

***Gel Filtration***: The relative molecular mass of the lectin was determined by gel filtration on Sephadex G-100 (1.2 cm × 100 cm) according to the method of Andrews [19]. The column was equilibrated with THS containing 0.2 M galactose to avoid any lectin matrix interaction. It was calibrated with bovine serum albumin (66,000), carbonic anhydrase (29,000) and cytochrome C (12,400).

***SDS-PAGE***: The apparent molecular mass of the subunits was determined by SDS-PAGE according to the method of Laemmli [20] using a 12.5 % (w/v) gel. Bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase

(29,000), trypsinogen (24,000), trypsin inhibitor (20,000) and lactalbumin (14,200) were used as reference proteins. Protein bands were visualised by Coomassie Brilliant Blue R-250.

**Isoelectric focusing**

Isoelectric focusing in polyacrylamide gels was performed according to Vesterberg [21] over the pH range 3-10. The gels were stained with Coomassie Brilliant Blue R-250. The isoelectric point was confirmed by density gradient single column isoelectric focussing [22].

**Carbohydrate content**

The total neutral sugar content was estimated by the phenol sulfuric acid method of Dubois et al. [23] using mannose as standard. 300 μg of *A. hirsuta* lectin in 400 μl water was mixed with 400 μl of 5 % w/v phenol and incubated for 10 min at room temperature. 2 ml concentrated sulphuric acid was then added to the mixture, allowed to cool for 20 min and the O.D. was measured at 490 nm.

**Amino acid Analysis**

The amino acid composition of the salt-free lectin was determined on an automated amino acid analyser (Hewlett Packard series 1050 with fluorescence detector). The samples were hydrolysed in 200 μl of 6 N HCl for 20 h at 110°C and then subjected to analysis. Cysteine [24,25] and tryptophan [26] contents were determined spectrophotometrically.

**Effect of metal ions**

The activity of the purified lectin was determined in the presence of 1, 5 and 10 mM of $Ca^{++}$, $Mn^{++}$, $Mg^{++}$ and also after dialysis against 10 mM of EDTA.

**Temperature and pH stability**

Effect of temperature on lectin stability was monitored in the range of 0°C to 80°C by incubating 25 μg of the lectin for 30 minutes at the respective temperature, rapidly cooling in ice and assaying for hemagglutinating activity. Effect of pH on lectin stability was determined with 25 μg lectin and adjusting to different pH values from 3-9 by adding the lectin into 1 ml of universal buffer and checking activity after 3 h and 24 h.

**Human blood group specificity**

The hemagglutination tests were carried out with a 3 % suspension of human erythrocytes of A, B and O blood group in THS.

**Atomic Absorption Spectroscopy**

The magnesium, manganese and calcium contents of the lectin sample were determined on an ATI UNICAM 929 AA Spectrometer at 285.10 nm, 279.48 nm and 422.7 nm respectively. Prior to analysis, the lectin was extensively dialysed against deionised water (to remove buffer salts) and centrifuged to remove any insoluble material (5mins, 8000 rpm).

**Circular dichroism measurements**

C.D. spectra were recorded on a Jasco J-500A spectropolarimeter. The spectrum for secondary structure determination was recorded by using a path length of 1.0 mm, a protein concentration of 2.33 μM, in 20 mM

phosphate buffer, pH 7.2 and the region scanned was from 190-250 nm. The secondary structure determination was done by using a program based on unintelligent search technique for minimum error, which utilised the standard spectra of fifteen reference proteins [27]. Spectrum was also recorded for the same concentration of protein saturated with 6.6 mM methyl α-galactose in the same wavelength region.

C.D. spectra for 13.58 μM lectin with and without sugar (36 mM) were recorded in the wavelength region from 250-300 nm using a path length of 10 mm.

**Fluorimetric studies**

Fluorescence measurements were made on a Perkin Elmer LS 5B fluorimeter, with slit width of 5 nm for both the monochromators. 2 ml samples were placed in a quartz cuvette at constant temperature (25°C). The concentration of 4-methyl umbelliferyl α-galactose (MeUmb α-Gal) was determined spectrophotometrically from its molar absorbance of $1.36 \times 10^4$ $M^{-1}cm^{-1}$ at 318 nm [28].

The fluorescent sugar solution (2.2 μM) was excited at 318 nm and spectra were recorded above 325 nm both in the absence and after each addition of *A. hirsuta* lectin (166 μM). A double reciprocal plot of fluorescence intensity ($F_0$ / $F_0$-$F_c$) versus lectin concentration 1/ [P] gave the fluorescence intensity at infinite protein concentration ($F_\infty$) where $F_0$ and $F_c$ are the values of fluorescence intensity in the absence of protein and at specific protein concentration. When log [P] was plotted against log [$F_0$-$F_c$ / $F_c$-$F_\infty$], the abscissa intercept yielded the $pK_a$ value for the interaction of MeUmb α-Gal with the *A. hirsuta* lectin. At the end of the titration a 50 μl aliquot of methyl α-galactose (Me α-Gal) was added to check the reversal of MeUmb α-Gal binding.

For determination of the number of binding sites, a fixed concentration of *A. hirsuta* lectin (2.5 μM) was incubated with varying concentrations of MeUmb α-Gal (0.25-9.5 μM) in a total volume of 2 ml at 25°C. The quenching in fluorescence of MeUmb α-Gal was measured after equilibrating the samples for 24 h. A linear standard curve for MeUmb α-Gal in THS at 25°C was obtained in the same concentration range (0.25-9.5 μM). The relative fluorescence in the absence and presence of lectin gave the amount of free and bound sugar, for analysis of the data according to Scatchard [29].

## RESULTS

The ammonium sulphate precipitated and dialysed seed extract when chromatographed on DEAE-cellulose yielded two protein peaks, both showing hemagglutination activity (Fig. II.1a); one in the flow through fractions and the other in the eluate. The minor impurity, in the DEAE-cellulose unbound lectin sample was removed by subjecting it to CM-Sephadex chromatography (Fig. II.1b). The DEAE unbound sample when chromatographed on guar gum (Fig. II.2a) gave a higher yield of the purified lectin (84 mg) as compared to CM-Sephadex (78 mg) but the associated color was not removed. However, when the seed extract was subjected to chromatography on cross-linked guar gum (prior to DEAE-cellulose chromatography) both the lectins co-eluted (Fig. II.2b).

The yield of the purified lectin obtained by sequential ion exchange chromatography was 78 mg from 100 g seeds, with a specific activity of 1.2 $\times 10^5$ U/mg. The purified lectin showed a single band on native PAGE at pH 4.5 (Fig. II.3a) and on IEF gel (Fig. II.3b). The molecular mass of the

native lectin was 60,000 as determined by gel filtration on Sephadex G-100 (Fig. II.4). The lectin showed two bands, of $M_r$ 15,800 and 14,200 on SDS-PAGE, in the presence and absence of β-mercaptoethanol (Fig. II.5). The isoelectric point of the lectin was 8.5 (Fig. II.3b) and the neutral sugar content 6.25 % (w/w). Amino acid analysis (Table I) revealed that the lectin is rich in glycine (15 %), has a high percentage of lysine (7 %) while low in sulphur containing amino acids (Met 1.6 % and cysteine absent). Hydrophobic amino acids (14 % aromatic, 27 % aliphatic and 1.6 % methionine) constitute a large part of the molecule. C.D. analysis showed that the *A. hirsuta* lectin has 6 % α helix, 32 % β sheet, 36 % β turns and 25 % random coil. The C.D. spectrum of the lectin in the far UV (Fig. II.6a) and near UV (Fig. II.6b) region in the presence of Me α-Gal showed that aromatic residues were affected on binding of Me α-Gal whereas, ligand-binding did not affect the polypeptide backbone of the lectin.

The lectin was found to be stable in the pH range 3-9 for 24 hours at 25°C. It was stable at 30°C for 6 h, lost 25 % of its activity at 40°C and total activity at 60°C after 30 min. The lectin activity was neither affected by $Ca^{++}$, $Mg^{++}$, $Mn^{++}$ nor by EDTA upto 10 mM concentration. Atomic absorption spectroscopy showed the lectin contains calcium (0.84 mole/mole). Human erythrocytes of the blood groups A, B and O were agglutinated by the *A. hirsuta* lectin with equal affinity.

The agglutination of rabbit erythrocytes by *A. hirsuta* lectin was not inhibited by glucose, mannose, lactose, rhamnose, xylose, fucose, raffinose, N-acetyl-glucosamine (GlcNAc), N-acetyl-mannosamine (ManNAc), mannosamine, glucosamine and stachyose upto 125 mM concentration (Table II). The minimum concentrations of Me α-Gal, p-nitrophenyl α-galactose (pNP α-Gal) and p-nitrophenyl α-N-acetyl-galactosamine (pNP α-GalNAc) required for inhibition were 3.25 mM, 0.156 mM and 0.250

mM respectively. The T-antigen disaccharide Gal β 1-3 GalNAc failed to inhibit the lectin activity (upto 30 mM concentration).

Fluorescence intensity of MeUmb α-Gal (2.2 μM) was totally quenched on titration with *A. hirsuta* lectin (166 μM) with no change in the emission maximum (Fig. II.7). This dramatic decrease was almost totally reversed upon the addition of Me α-Gal (0.05 M) to the *A. hirsuta* lectin-MeUmb α-Gal complex. The data obtained by titration gave value of the association constant $K_a$ as $3.3 \times 10^5$ $M^{-1}$.

The changes in fluorescence intensity of MeUmb α-Gal with a fixed concentration of *A. hirsuta* lectin were subjected to Scatchard analysis. The Scatchard plot obtained with MeUmb α-Gal yielded a straight line with intercept 1.52 on the X axis and slope ($K_a$) obtained by the Scatchard plot was $3.5 \times 10^5$ $M^{-1}$ (Fig. II.8).

**Table I: Amino acid composition of the lectin from seeds of *Artocarpus hirsuta* and other *Artocarpus* species.**

| Amino acid residue | No. of residues per molecule in *A. hirsuta* lectin | Percentage in following species A | B* | C* | D° | E* | F° |
|---|---|---|---|---|---|---|---|
| Asx | 51 | 10.0 | 8.7 | 8.7 | 7.7 | 9.2 | 8.7 |
| Thr | 33 | 6.0 | 6.9 | 8.1 | 5.3 | 7.0 | 7.4 |
| Ser | 24 | 4.4 | 9.3 | 8.7 | 5.3 | 8.7 | 7.0 |
| Glx | 40 | 8.0 | 8.1 | 6.4 | 7.6 | 7.0 | 8.6 |
| Gly | 76 | 15.0 | 14.5 | 13.9 | 5.6 | 14.8 | 12.4 |
| Ala | 26 | 5.0 | 3.5 | 3.5 | 2.7 | 2.3 | 4.9 |
| Val | 46 | 9.0 | 9.3 | 9.3 | 5.0 | 9.1 | 5.7 |
| Met | 8 | 1.6 | 0.6 | 0.6 | 0.2 | 1.2 | 1.2 |
| Ile | 36 | 7.0 | 5.8 | 6.4 | 4.8 | 7.0 | 6.9 |
| Leu | 31 | 6.0 | 5.8 | 5.8 | 5.1 | 6.4 | 6.2 |
| Tyr | 31 | 6.0 | 4.6 | 4.6 | 4.4 | 7.4 | 5.8 |
| Phe | 36 | 7.0 | 6.4 | 7.0 | 6.7 | 6.9 | 7.3 |
| His | 4 | 0.7 | 0.6 | 1.2 | 1.1 | 0.7 | 1.7 |
| Lys | 35 | 6.8 | 5.8 | 6.4 | 4.8 | 6.4 | 6.7 |
| Arg | 9 | 1.8 | 1.2 | 2.3 | 2.5 | 1.4 | 2.4 |
| Pro | 26 | 5.0 | 5.2 | 4.6 | - | 4.5 | 5.8 |
| Cys§ | 0 | 0.0 | 1.2 | 0.0 | 0.3 | ND | ND |
| Trp# | 4 | 1.0 | 1.2 | 1.2 | 6.3 | ND | 1.4 |

§ Cysteine was determined by the method of Ellman [15] and Cavallini [16]
# Tryptophan was determined by the method of Spande and Witkop [17].
ND – Not Determined
A : *A. hirsuta* lectin B : *A. champeden* lectin C : *A. tonkinensis* lectin
D : *A. lakoocha* lectin E : *A. integrifolia* lectin F : *A. incisa* lectin
* Adapted from ref. no. 4
¤ Adapted from ref. no. 8
° Adapted from ref. no. 5

**Table II: Sugar Inhibition of *Artocarpus hirsuta* lectin**

| Inhibitor* # | Min conc. of sugar causing total inhibition (mM) |
|---|---|
| Galactose | 62.5 |
| Galactosamine | 125 |
| N-acetyl-galactosamine | 62.5 |
| 2-deoxygalactose | 125 |
| Methyl α-Gal | 3.25 |
| Methyl β-Gal | NI° |
| pNP α-Gal | 0.156 |
| pNP β-Gal | NI°° |
| pNP α-GalNAc | 0.250 |
| pNP β-GalNAc | NI°° |
| Methyl α-Man | NI° |
| Methyl α-Glc | NI° |
| Stachyose | NI° |
| Lactose | NI° |
| Gal β1-3 GalNAc | NI°°° |

NI: Non inhibitory at the highest concentration, ° = 125 mM, °° = 2.5 mM, °°° = 30 mM.

* All the sugars used are of D configuration.

# The following sugars were non-inhibitory at 125 mM; glucose, mannose, rhamnose, xylose, L-fucose, raffinose, glucosamine, mannosamine, N-acetylglucosamine, N-acetylmannosamine.

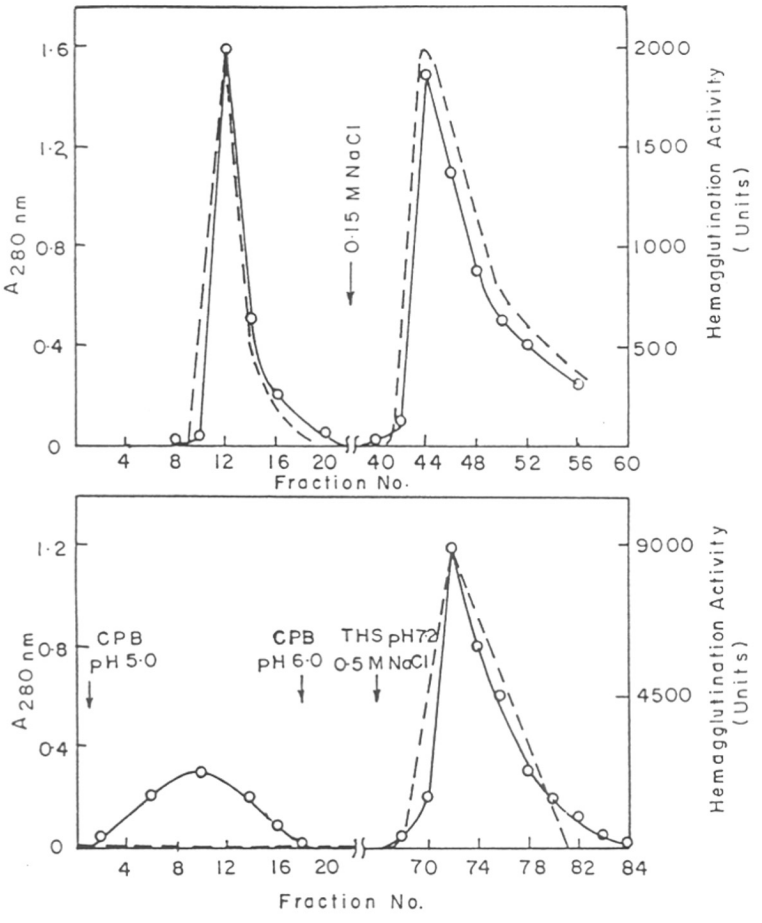


**Fig. II.1:**

**Elution profile of *A. hirsuta* lectin: (–) $A_{280}$ nm and (---) hemagglutination titre.**

a) Ammonium sulphate precipitated seed extract loaded on DEAE-cellulose. The column was washed with Tris-HCl buffer, pH 7.2, till $A_{280}$ nm reached the baseline. The protein was then eluted using 0.150 M NaCl (THS). Fractions of 2ml were collected.

b) DEAE-Cellulose unbound protein loaded on CM-Sephadex. The column was washed with CPB pH 5.0, CPB pH 6.0 and phosphate buffer pH 7.2. The protein was eluted with phosphate buffer containing 0.5 M NaCl in fractions of 2ml.

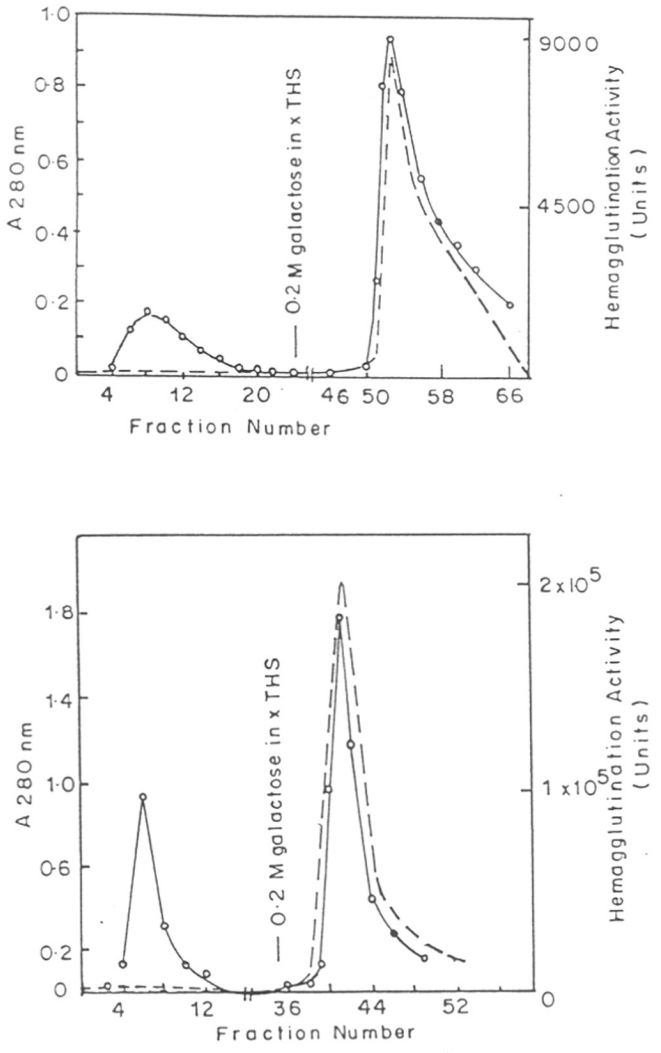


**Fig. II.2:**

**Elution profile of *A. hirsuta* lectin: (–) $A_{280}$ nm and (---) hemagglutination titre.**

a) DEAE-cellulose unbound protein loaded on cross-linked guar gum. The column was washed with THS till $A_{280}$ nm reached the baseline. The lectin was then eluted using 0.2 M galactose in THS. Fractions of 2ml were collected.

b) Ammonium sulphate precipitated seed extract loaded on cross-linked guar gum. Column washing and elution was as in (a).

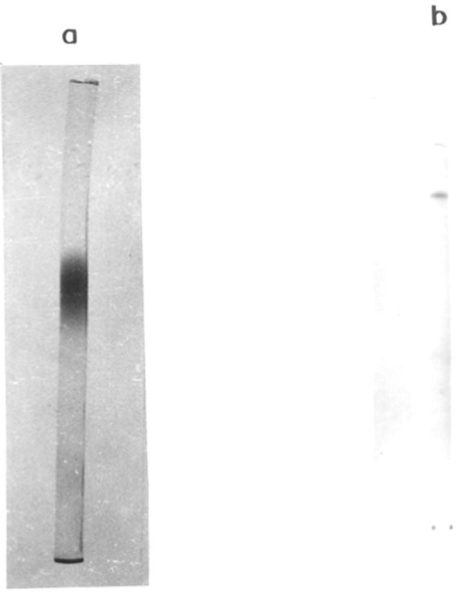


**Fig. II.3:**

**Electrophoresis of the purified *Artocarpus hirsuta* lectin:**
**a) Native-PAGE:** The lectin (50 μg) was electrophoresed in a 7.5 % (w/v) polyacrylamide gel, at pH 4.5, current 4 mA/ tube, and the protein was visualised with Coomassie Brilliant Blue G-250.
**b) IEF-PAGE:** The lectin (100 μg) was focussed for 1600 Vh, in a 7.5 % (w/v) polyacrylamide gel using wide range ampholines (pH range 3.0-10.0) and the gels were stained with Coomassie Brilliant Blue R-250.

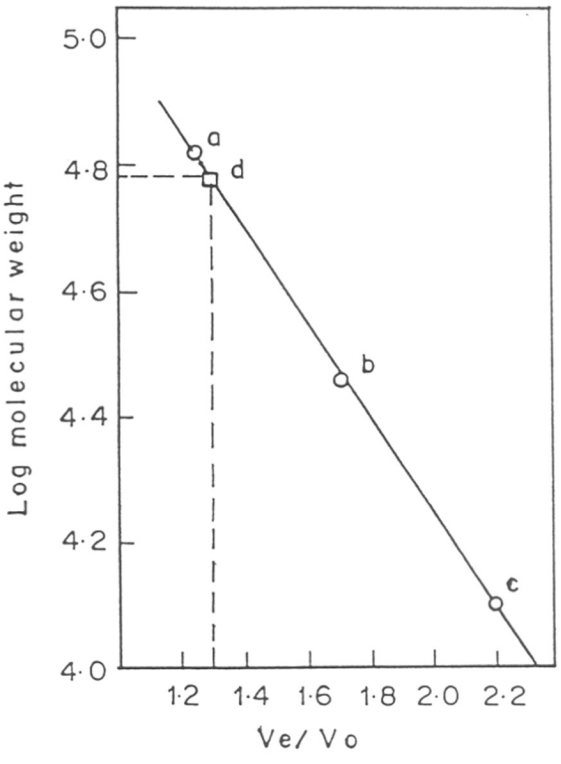


**Fig. II.4:**

**Molecular weight determination of the purified *A. hirsuta* lectin:**
Gel filtration: Sephadex G-100 column (1.2 cm × 100 cm) was equilibrated with THS (pH 7.2) containing 0.2 M galactose and calibrated with (a) bovine serum albumin (66,000) (b) carbonic anhydrase (29,000) (c) cytochrome C (12,400). $V_0$ is the void volume, $V_e$ the elution volume and (d) indicates the purified lectin.

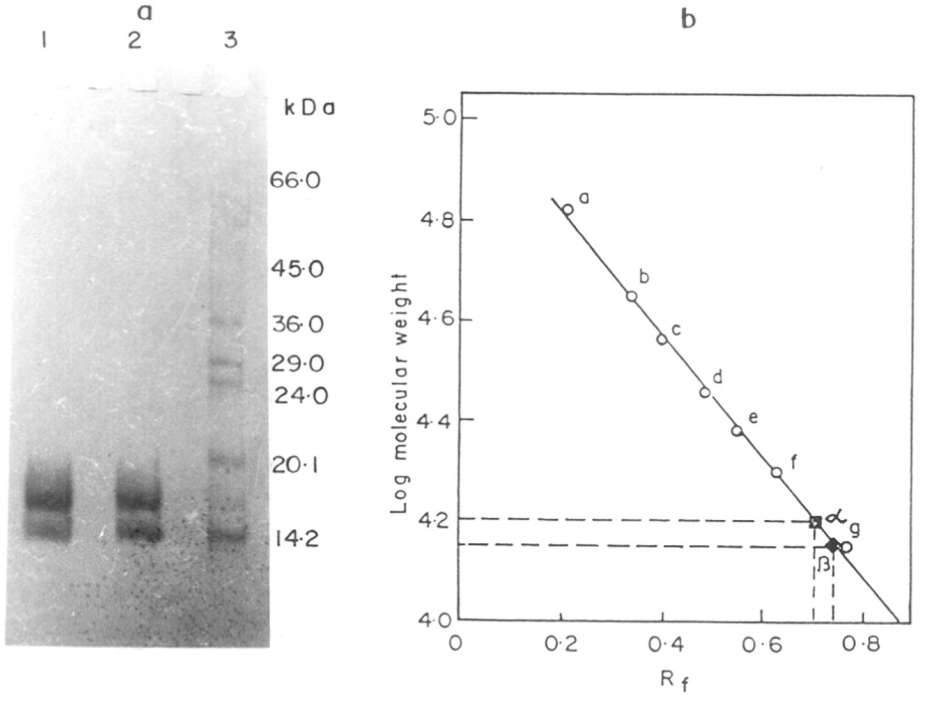


**Fig. II.5:**

**SDS-PAGE of the purified *A. hirsuta* lectin:**

**a)** The lectin (50 μg) was electrophoresed using a 12.5% (w/v) polyacrylamide gel containing 0.1 % SDS according to Laemmli at constant voltage (200 V) for 4.5 h and stained with Coomassie Brilliant Blue R-250. Lane 1 – lectin in the presence of β-mercaptoethanol. Lane 2 – lectin in the absence of β-mercaptoethanol. Lane 3 - molecular weight markers.

**b) Relative** mobilities of the reference proteins were plotted against the log molecular weight. Reference proteins used were (a) Bovine serum albumin $M_r$ 66,000, (b) Egg albumin $M_r$ 45,000, (c) Glyceraldehyde 3-phosphate dehydrogenase $M_r$ 36,000, (d) Carbonic anhydrase $M_r$ 29,000, (e) Trypsinogen $M_r$ 24,000, (f) Trypsin inhibitor $M_r$ 20,100, (g) α-lactalbumin $M_r$ 14,200, (■) α subunit $M_r$ 15,800 and (♦) β subunit $M_r$ 14,200.

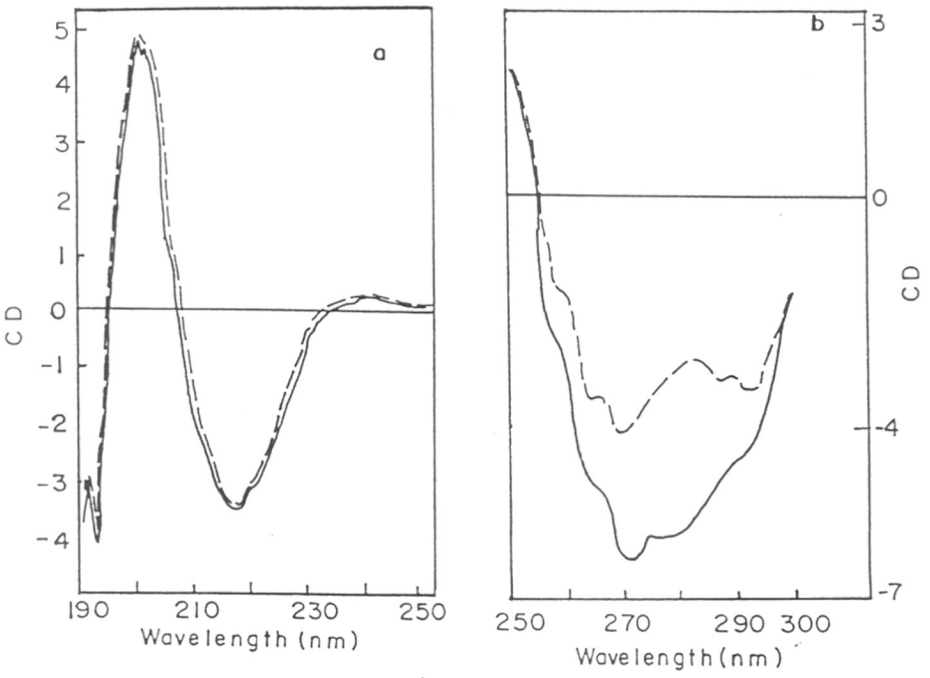


Fig. II.6:

**C.D. spectra of the *A. hirsuta* lectin in the absence (- -) and presence of saturating concentrations of methyl α-galactose (—):**
a) Far UV C.D. spectra were recorded in a 1 mm pathlength cell. No change in the far UV C.D. spectrum of the lectin was brought about by the sugar, ruling out conformational changes in the polypeptide backbone.
b) Near UV C.D. spectra were recorded in a 10 mm pathlength cell. The spectrum was altered on ligand binding.

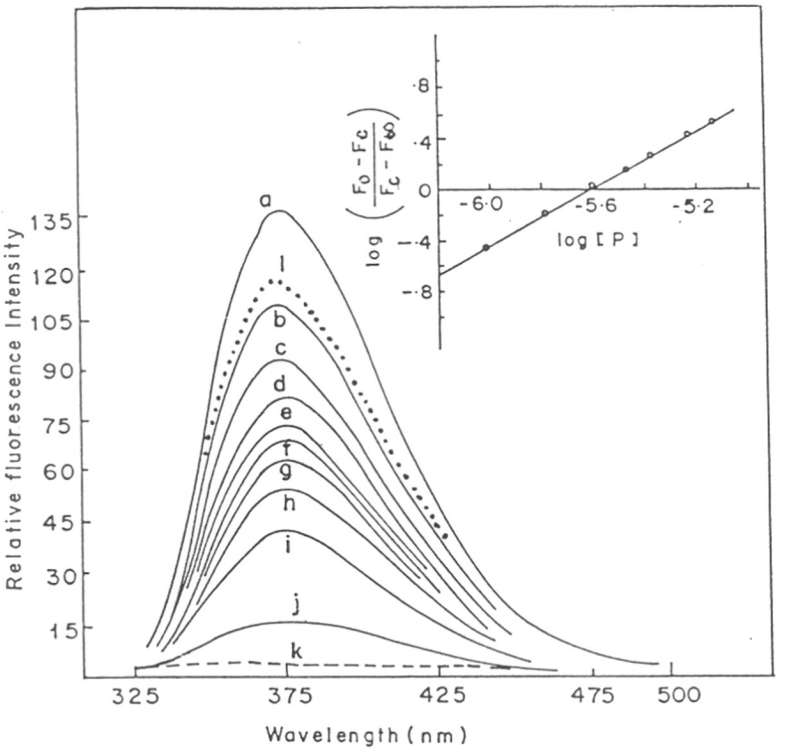


**Fig. II.7:**

**Fluorescence quenching of methylumbelliferyl α-galactose on titration with *A. hirsuta* lectin:** A 2.2 μM solution of MeUmb α-Gal in Tris buffered saline was titrated at 25°C with 166 μM lectin in the same buffer. Curve a represents the fluorescence spectrum due to the sugar alone, curve b-j represents spectra obtained upon successive additions of the *A. hirsuta* lectin, curve k (--) represents the spectrum for the buffer alone and curve l (···) represents reversal of fluorescence quenching, on addition of Me α-Gal. The inset gives a graphical representation for the determination of the association constant. $F_0$ and $F_\infty$ are the fluorescence intensities of free ligand and that bound to the lectin at infinite concentration. $F_c$ is the corrected value of fluorescence intensity at any point of titration. [P] is the protein concentration in molarity.

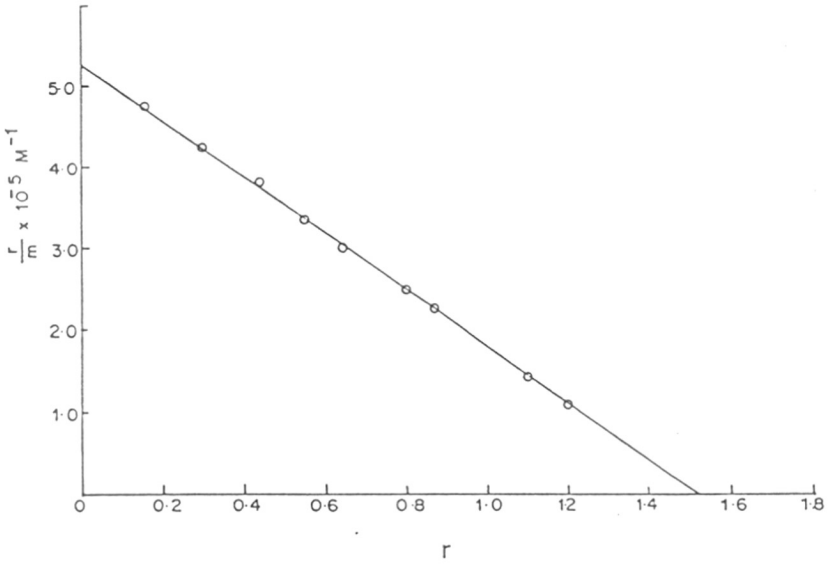


**Fig. II.8:**

**Scatchard plot for the binding of methylumbelliferyl$\alpha$-galactose to *A. hirsuta* lectin:** The fluorescence of the ligand protein mixture was compared with respect to that of equal concentration of ligand in the absence of protein; r is the ratio of moles of sugar bound per mole of lectin and m is the concentration of free sugar. The slope $K_a$ is $3.5 \times 10^5$ $M^{-1}$ and the intercept on the X axis is 1.52, binding sites per molecule.

## DISCUSSION

DEAE-Cellulose chromatography carried out at pH 7.2, separated out the acidic proteins as well as most of the colour present in the seed extract. The pooled fractions of the washing showing hemagglutination activity contained a minor impurity. Complete removal of color and this impurity was achieved after CM-Sephadex chromatography.

As the seed extract showed inhibition by α-galactose, affinity chromatography on guar gum was attempted to achieve single step purification. But co-elution of two lectins with comparable affinity for α-linked galactose, did not allow single step purification. The major difference between the two lectins was their behaviour on DEAE-cellulose at pH 7.2. When guar gum was used instead of CM-Sephadex, as a second step of purification after DEAE-cellulose, a significant amount of color was retained. Therefore affinity chromatography was not preferred and the lectin was purified by two sequential ion exchange chromatographies.

The purification procedure yielded an electrophoretically homogenous lectin and SDS-PAGE showed two closely spaced bands of molecular mass 15,800 and 14,200 in the presence and absence of β-mercaptoethanol. Incorrect estimation of the molecular mass on gel filtration due to retardation upon lectin-matrix interaction has been reported for some proteins [30]. To overcome any such interaction on Sephadex G-100, gel filtration was carried out in the presence of 0.2 M galactose. The purified lectin has molecular mass of 60,000 by gel filtration and therefore is a heterotetramer of the $\alpha_2\beta_2$ type consisting of two pairs of non-identical subunits held non-covalently. The lectin is a glycoprotein with 6.25 % neutral sugar content and is basic with pI of 8.5.

The lectin showed stability over a wide pH range similar to other plant lectins. *A. hirsuta* lectin is a metalloprotein but does not require metal ions for its activity. *Maclura pomifera* lectin is an example of a lectin not requiring metal ions for activity [31] while Con A [32], soybean [33] and *Dolichos biflorus* [34] lectins require $Mn^{++}$ and /or $Ca^{++}$, respectively. Similar to other *Artocarpus* lectins (Table I), the glycine and valine content of the *A. hirsuta* lectin is high while the content of sulphur containing amino acids and tryptophan is low.

The lectin showed specificity for the α-anomeric derivatives of galactose. The inhibition by Me α-D Gal, pNP α-Gal and pNP α-GalNAc was 20, 400 and 250 fold more than that of galactose respectively. Me α-D Gal was over 40 fold as effective as an inhibitor compared to Me β-D Gal. The higher specificity could be due to the fact that the aglycone at C-1 being axial is accessible to the lectin-binding site whereas the aglycone moieties in the β anomers, present in the equatorial position may sterically hinder the lectin carbohydrate interactions. The better inhibition caused by pNP α-Gal and pNP α-GalNAc over Me α-D Gal can possibly be due to the aromatic aglycone. Sugars having aromatic aglycones show greater affinity than those with alkyl aglycones, indicating the existence of a hydrophobic region at/adjacent to the monosaccharide-binding site [35]. The *A. hirsuta* lectin was able to distinguish between the α and β anomers of aromatic as well as alkyl glycosides, indicating specificity similar to other galactose specific lectins like peanut lectin [36] and *Ricinus communis* agglutinin [37] where the preference for the α or β anomer is retained on going from an alkyl to an aromatic aglycone. Galactose specific *Griffonia simplicifolia* lectin [32] could not distinguish between the α and β aromatic aglycones.

Stachyose, the C-1 hydroxyl of which is substituted by a sugar in place of the aglycone moiety, was ineffective as an inhibitor. Relative affinities of galactose, galactosamine, N-acetyl-galactosamine and 2-deoxygalactose were similar indicating that the hydroxyl group at C-2 had little influence on binding. Inversion of C-4 hydroxyl group as in glucose or C-2 and C-4 as in mannose made these sugars ineffective as inhibitors. Fucose (6-deoxygalactose) was non-inhibitory indicating a possible role for the primary hydroxyl group at C-6. Schematic representation of the binding site of *A. hirsuta* lectin is shown in Figure II.9.

Circular dichroism used in the structural analysis of proteins correlates the spectra with the secondary structure of protein. The predominance of β conformation in several lectin molecules has been shown using C.D. viz Con A [38], *Dolichos biflorus* lectin [39], pea lectin [40] and *B. simplicifolia* lectin [41]. Me α-Gal, the most complementary ligand for the lectin, caused alteration in the near UV (aromatic) C.D. bands, indicating the possible involvement of aromatic amino acids in saccharide binding.

Fluorescence of MeUmb α-Gal was completely quenched on titration with the *A. hirsuta* lectin, indicating a change in the environment of the umbelliferyl moiety on binding to the lectin. The relative fluorescence of MeUmb galactopyranosides decreases as a function of solvent polarity [42], hence the region in and around the binding site may be hydrophobic. Quenching of MeUmb Gal has been observed for wheat-germ agglutinin [43], Con A [44], *Momordica charantia* lectin [45] and rice lectin [46]. The complete reversal of quenching observed when Me α-Gal is added to the lectin-fluorescent sugar complex indicated that quenching with MeUmb α-Gal is sugar specific. $K_a$ value of MeUmb α-Gal by Scatchard, $3.5 \times 10^5\ M^{-1}$ was in good agreement with that obtained by

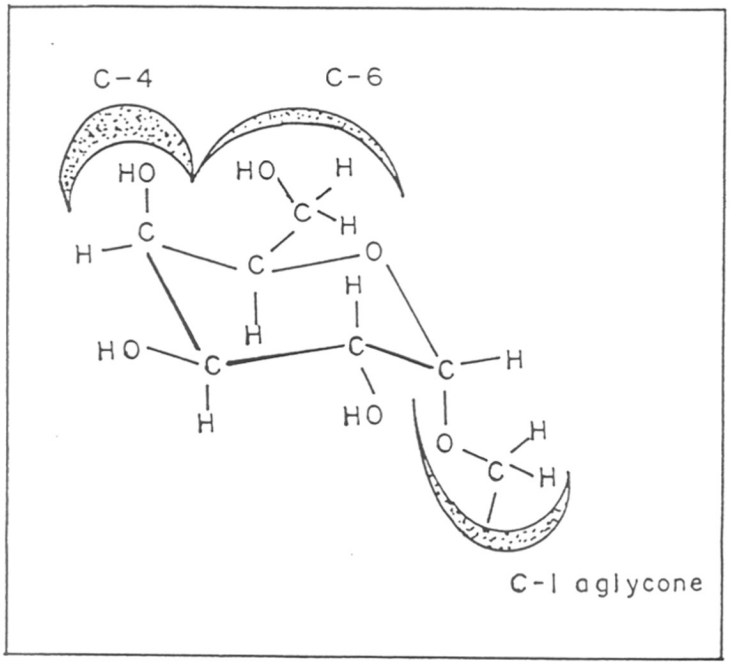


Fig. II.9:

Schematic representation of the *A. hirsuta* lectin binding site.

titration 3.3 × $10^5$ $M^{-1}$. The linearity of the Scatchard plot at fractional saturation and lack of deviation at high saturation excluded the existence of high and low affinity sites and indicated the absence of interaction between the two sites. *Ricinus communis agglutinin* [47] and *Momordica charantia* lectin [48] are examples of tetrameric lectins possessing two binding sites.

In conclusion, the *A. hirsuta* basic lectin is a $\alpha_2\beta_2$ type tetramer possessing two binding sites per molecule and having high affinity for methyl α-galactose.

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# CHAPTER III

# CHEMICAL MODIFICATION STUDIES ON *ARTOCARPUS HIRSUTA* LECTIN

## SUMMARY

Chemical modification studies on purified *A. hirsuta* lectin revealed the involvement of lysine and tyrosine in sugar binding. Lysine residues were modified using four different reagents and protection from inactivation by methyl α-galactose as well as recovery of activity by decitraconylation confirmed the involvement of lysine in the activity of the lectin. Tyrosine modification by N-acetylimidazole did not result in any inactivation, however total loss of activity was observed upon modification with tetranitromethane. Detailed investigation revealed that the positive charge of the ε–amino group of lysine and the phenyl ring of tyrosine are important for sugar binding activity of the lectin. These studies also showed that tryptophan, carboxylate, histidine, serine and arginine residues have no role in the lectin activity.

## INTRODUCTION

To understand the phenomenon of interaction between lectins and carbohydrates, knowledge of the reactive groups that participate in the interaction is essential. Identification of specific amino acids within the active site of a biologically active protein elucidates the relationship between its structure and the role played by the amino acid side-chains in its activity. Single crystal X-ray diffraction is an excellent method to determine these residues but it is often difficult to obtain good quality crystals of macromolecules. Other than site-directed mutagenesis, chemical modification is an alternative, which provides reasonable information about the residues involved in saccharide binding.

Lectins have been isolated from six species of *Artocarpus* but very little information is available about the amino acid residues involved in their sugar binding site. In jacalin, tyrosine and lysine have been implicated in the saccharide binding [1] whereas in *A. lakoocha* lectin, involvement of histidine, tyrosine and tryptophan has been demonstrated [2]. However no report exists on the number of essential residues involved in the lectin activity. The present chapter describes chemical modification studies to understand the structure-function relationship of the *A. hirsuta* lectin.

## MATERIALS AND METHODS

### *Materials*

Succinic anhydride, citraconic anhydride, acetic anhydride, N-acetyl imidazole, Woodward's reagent K, 2,4,6-Trinitrobenzenesulphonic acid, diethylpyrocarbonate,phenylmethylsulphonylfluoride,N-bromosuccinimide, 2-hydroxy 5-nitrobenzylbromide, phenyl glyoxal, 5,5'-dithiobis 2-nitrobenzoic acid, imidazole, guar gum and methyl α-galactose were obtained from Sigma Chemical Company (St. Louis, U.S.A). Sephadex G-100 and G-25 was purchased from Pharmacia (Uppasala, Sweden). Formaldehyde and sodium borohydride were obtained from BDH (India). Tetranitromethane was synthesised according to Liang [3]. *A. hirsuta* lectin was purified as described in chapter II.

### *Methods*

**Hemagglutination assay:** The lectin samples were serially diluted in 10 mM Tris-HCl buffer, pH 7.2, containing 150 mM NaCl (THS), in a microtitre plate and equal quantity of a 3 % suspension of rabbit

erythrocytes was added. After incubation for one hour at room temperature, the agglutination was scored visually. Taking the titre value of the native lectin to be 100 %, percentage residual agglutination of the modified lectin samples were calculated from their respective titre values.

**Protein determination**: Protein concentration was determined by the method of Lowry et al. [4] using bovine serum albumin as standard.

*CHEMICAL MODIFICATION STUDIES*

**Modification of Serine with Phenylmethylsulphonyl fluoride (PMSF)**

The lectin (300 μg) in 50 mM phosphate buffer, pH 7.2 was incubated with 5 mM PMSF, at room temperature, for 60 minutes [5]. Aliquots were removed at 15 min intervals, the excess reagent removed by dialysis and residual activity determined. Lectin sample incubated in the absence of PMSF served as control.

**Modification of Cysteine with 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB).**

The lectin (300 μg) in 50 mM phosphate buffer, pH 7.2, was incubated with 0.1 mM of DTNB (effective concentration) at room temperature for one hour. Aliquots were removed at different time intervals and the residual activity determined. The modification reaction was also followed by monitoring the increase in absorbance at 412 nm and the number of sulfhydryl groups modified were calculated using a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ [6].

**Modification of Arginine with Phenylglyoxal**

Arginine residues were modified with phenyl glyoxal by the method of Takahashi [7]. The reagent was prepared in methanol. The lectin (300 μg), in 50 mM phosphate buffer, pH 8.0, was treated with varying concentrations of phenylglyoxal (0.5-3.0 mM) for 30 minutes at 30°C. Excess reagent was then removed by dialysis and the residual hemagglutination activity determined. Lectin sample incubated in the absence of phenylglyoxal served as control. The methanol concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the lectin during the incubation period.

**Modification of Carboxylate groups with Woodward's Reagent K (WRK)**

The modification of carboxylate residues with WRK was carried out by incubating 400 μg of the purified lectin, in 50 mM phosphate buffer pH 7.0, with different concentrations (0.01-0.3 mM) of WRK. Aliquots were removed after every 10 min and the reaction stopped by addition of 100 mM acetate buffer, pH 4.5. The reaction mixture was dialysed to remove excess reagent and the residual activity determined. Lectin samples incubated in the absence of WRK served as control. The number of carboxylate groups modified were determined spectrophotometrically, at 340 nm, by assuming a molar absorption coefficient of 7000 $M^{-1}cm^{-1}$ [8].

**Modification of Tryptophan**

***Reaction with 2-hydroxy-5-nitrobenzylbromide (HNBB):*** This was done according to the method of Horton and Koshland [9]. HNBB was prepared in dry acetone. *A. hirsuta* lectin (300 μg), in 100 mM sodium acetate buffer, pH 5.0, was incubated with 2 mM HNBB at 30°C for 30 minutes. After the

incubation period the excess reagent was removed by gel filtration on Sephadex G-25 (equilibrated with 100 mM acetate buffer pH 5) and the residual activity was determined. The number of hydroxynitrobenzyl groups introduced was determined using a molar absorption coefficient of 18,450 $M^{-1}cm^{-1}$ at 410 nm. Lectin samples incubated in the absence of HNBB served as control. The acetone concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the lectin during the incubation period.

***Reaction with N-bromosuccinimide (NBS)***: The lectin (300 μg), in 100 mM sodium acetate buffer pH 5.0, was titrated with a total of 0.02 mM NBS, prepared in the same buffer. The reagent was added in five installments and the reaction was monitored spectrophotometrically by measuring the decrease in absorbance at 280 nm. The number of tryptophan residues modified were determined by assuming a molar absorption coefficient of 5500 $M^{-1}cm^{-1}$ [10]. The residual activity was determined by hemagglutination.

**Modification of Lysine**

***Estimation of free amino groups with Trinitobenzenesulphonic acid (TNBS):*** The reaction mixture containing 0.5 ml of lectin (150 μg) and 0.5 ml 4% (w/v) sodium bicarbonate was incubated with 50 μl of 0.5 % TNBS at 37°C, in the dark, for 2 hours. The reaction was terminated by adjusting the pH to 4.5 with 0.25 ml HCl (1 N) and 0.5 ml of 10 % SDS was added to dissolve the precipitate formed [11]. The number of free amino groups was determined, spectrophotometrically by assuming a molar absorption coefficient of 9950 $M^{-1}cm^{-1}$ for trinitrophenylated lysine at 335 nm.

***Acetylation:*** *Acetylation* was performed as described by Fraenkel-Conrat [12]. To 1.5 mg of *A. hirsuta* lectin, in 3 ml saturated sodium acetate, six

aliquots of acetic anhydride (0.1-1 mM) were added over the course of one hour. After each addition, an aliquot was removed and assayed for hemagglutination activity. The number of amino groups modified at the end of the reaction by acetic anhydride were estimated by determining the number of free amino groups using TNBS and subtracting it from the total number of free amino groups as determined earlier.

***Citraconylation and decitraconylation***: The amino groups of *A. hirsuta* lectin were reversibly blocked by citraconic anhydride according to Dixon and Perham [13]. Citraconic anhydride was diluted in dioxane and the concentration of the diluted reagent was 110 mM. Purified *A. hirsuta* lectin (1.5 mg) in 5 ml sodium carbonate buffer (100 mM), pH 8.5, was treated at room temperature with a total of 25 µl of citraconic anhydride. After each addition an aliquot was removed and assayed for hemagglutination as well as saccharide-binding activity and free amino groups. Control consisted of lectin samples incubated under identical conditions without citraconic anhydride. The number of amino groups in the native and modified lectin samples were estimated by the TNBS method of Habeeb [11.] Decitraconylation was achieved by incubating the modified lectin samples, at pH 4.0 and 30°C for one hour followed by determining the activity.

***Succinylation***: This was carried out by the method of Habeeb et al. [14]. Lectin (1 mg), in 100 mM sodium carbonate buffer pH 8.5, was incubated with varying concentrations of succinic anhydride (0.5-3.0 mM) prepared in dioxane. The residual activity and the number of amino groups modified were determined as described above.

***Reductive methylation:*** This was carried out as described by Means and Feeney [15]. To 1 ml of the lectin (0.3 mg/ml) in 200 mM borate buffer pH 9.0, at 0°C, 0.1 ml sodium borohydride solution (0.5 mg/ml) was added, followed by 6 aliquots (5 µl each) of 0.35 % (v/v) formaldehyde at 10

minute intervals. The procedure was repeated using 3.5 % formaldehyde. At the end of the reaction, the residual hemagglutination activity and the number of amino groups modified were determined as described above. Lectin sample incubated in the absence of formaldehyde served as control.

**Modification of Tyrosine**

***Reaction with N-acetylimidazole (NAI)***: This was performed as described by Riordan et al. [16]. To 300 μg of the lectin, in 50 mM phosphate buffer pH 7.5, 1000 fold molar excess of NAI was added and incubated at 30°C for 30 min followed by estimation of the residual activity. The lectin incubated in the absence of N-acetylimidazole served as control. The tyrosine residues modified were determined spectrophotometrically, using a molar absorption coefficient of 1160 $M^{-1}cm^{-1}$ at 278 nm. The NAI mediated modification of lysine residues was determined by estimating the number of amino groups before and after the modification reaction, by the TNBS method of Habeeb [11].

***Reaction with Tetranitromethane (TNM):*** Nitration was carried out according to the method of Sokolovsky [17]. *A. hirsuta* lectin (500 μg) in 50 mM Tris–HCl buffer pH 8.0, was treated with a total of 0.4 mM of TNM (freshly diluted with 95% ethanol). The reaction mixture was allowed to stand for 1 hour, passed through Sephadex G-25 equilibrated with 50 mM Tris-HCl buffer, pH 8.0 and the hemagglutination as well as saccharide-binding activity were determined. Lectin incubated in the absence of TNM was taken as control. The nitrotyrosine residues were quantitated, at 428 nm, using a molar absorption coefficient of $4100M^{-1}cm^{-1}$

**Modification of histidine**

***Photo-oxidation***: The reaction was carried out by exposing, 300 μg/ml of the purified lectin in 20 mM phosphate buffer pH 7.0, with different concentrations of Methylene Blue (0.1-0.5%) to a 200 W light bulb held at a distance of 10 cm for 30 minutes at 30°C [18]. Subsequently the residual activity was determined. Lectin samples treated under identical conditions, in the dark, served as control.

***Modification with Diethylpyrocarbonate (DEP):*** 300 μg of lectin in 1 ml of 20 mM phosphate buffer, pH 7.0 was treated with varying concentrations of DEP (1-5 mM), freshly prepared in absolute ethanol. Modification of histidine residues was monitored spectrophotometrically by measuring the increase in absorbance at 240 nm as described by Ovadi et al. using a molar absorption coefficient for carbethoxyhistidine of 3200 $M^{-1}cm^{-1}$ [19]. The ethanol concentration in the reaction mixture did not exceed 2 % (v/v) and had no effect on the activity and stability of the lectin during the incubation period. Lectin samples incubated in the absence of DEP served as control.

The DEP concentration in the diluted samples was determined by mixing an aliquot of the diluted sample with 3 ml of 10 mM imidazole buffer, (pH 7.5) followed by monitoring the increase in absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated using a molar absorption coefficient of 3000 $M^{-1}cm^{-1}$ [20]. The concentration of the diluted stock of DEP was 100 mM.

Decarbethoxylation of the DEP modified protein modified with DEP was carried out according to Miles [21]. The DEP treated samples were incubated with hydroxylamine hydrochloride (50 mM–1000 mM) at pH 5,6 and 7 at 25°C for 1-16 h and the hemagglutination activity was determined.

**Saccharide Binding**

Saccharide binding of the native and modified *A. hirsuta* lectin was monitored by following their retention on cross-linked guar gum. Native and modified lectin samples (1 mg/ml) were loaded on a cross-linked guar gum column (1 cm × 5 cm) equilibrated with Tris buffer saline pH 7.2 (THS). The column was washed with the same buffer and the bound lectin was eluted with 0.2 M galactose. Percentage binding of the modified lectin was calculated by assuming the amount of native lectin retained as 100 percent.

**Ligand protection**

This was carried out by pre-incubating the lectin with 100 mM methyl α-galactose (Me α-D Gal) followed by treatment with various modifying reagents. Both modified and unmodified protein samples were dialysed, the residual activity estimated and the number of residues modified determined.

**Circular dichroism**

C.D. spectra, in the range 190-250 nm were recorded for native and modified lectins at a protein concentration of 2.5 μM (2 ml), using Jasco J-710 spectropolarimeter at 25°C using a 1 cm path length cell. The C.D. spectrum was recorded in 100 mM carbonate buffer, pH 8.5 for the citraconylated and succinylated lectins and in 50 mM Tris-HCl buffer, pH 8 for the nitrated lectin.

## RESULTS

***Modification of lysine residues*:** Modification of the amino groups of the *A. hirsuta* lectin by acetic, succinic and citraconic anhydrides led to a loss of its sugar-binding and hemagglutinating activity. The loss of activity was concentration dependent. A plot of residual activity of the lectin versus the number of amino groups modified by citraconylation and succinylation indicated that four amino groups per molecule were essential for activity (Fig. III.1a,b). Decitraconylation of the inactivated lectin, at pH 4.0, brought about complete restoration of activity in one hour (Table I). Citraconic anhydride and succinic anhydride mediated inactivation could be prevented by incubating the lectin with excess Me α-Gal prior to the modification reaction. Molecular mass of the succinylated and citraconylated lectin, determined by gel filtration showed no significant change (Fig. III.2). The peptide backbone structure of the lectin on lysine modification did not undergo any gross change, as revealed by C.D. analysis (Fig. III.3). However, reductive methylation of the lectin did not result in any loss of hemagglutination activity even after ten amino groups were modified (Table I).

***Modification of tyrosine residues*:** Although acetylation of *A. hirsuta* lectin by NAI, resulted in the modification of five tyrosine residues, it had no effect on the hemagglutination activity (Table II). However, nitration of the lectin with TNM led to 90 % inactivation. No loss of activity was observed in the control samples. A plot of percent residual activity against number of tyrosine residues modified showed that loss of activity resulted from the modification of four tyrosine residues per molecule of the lectin (Fig. III. 4a). Pre-incubation of the lectin with excess Me α-Gal, resulted in 88 %

protection from inactivation. Moreover the C.D. spectra of the native and modified lectins (Fig. III.4b) showed no major change in the secondary structure of the lectin.

***Modification of histidine residues:*** Photo-oxidation of histidine did not lead to any inactivation of the lectin. However modification by DEP resulted in a total loss of activity with the concomitant modification of four histidine residues per molecule of the lectin. The DEP modified inactivation could not be reversed by incubation in the presence of varying concentrations of hydroxylamine hydrochloride, at different pH values for different time intervals.

Modification of the tyrosine residues (by following decrease in absorbance at 278 nm) and estimation of the lysine residues modified using TNBS, showed that DEP modified four lysine residues but did not modify tyrosine. Carbethoxylation carried out by first blocking the amino groups with citraconic anhydride, then modifying histidine with DEP and finally checking activity after decitraconylation, did not result in any loss of activity.

***Modification of arginine, serine, carboxylate and tryptophan residues*:** Modification of arginine residues by phenyl glyoxal and serine residues by PMSF did not cause any loss of lectin activity (Table II). WRK modified four carboxylate residues with no loss of activity. Modification of tryptophan (0.4 residues with HNBB and one with NBS) did not lead to any loss of activity.

**Table I: Effect of amino group modification of *A. hirsuta* lectin**

| Reagent | No. of residues modified /molecule | Guar gum binding activity | Residual Hemagglu -tinating activity % | Mr |
|---|---|---|---|---|
| Acetic anhydride | 16 | 0 | 10 | Unchanged |
| Succinic anhydride | 16 | 0 | 10 | Unchanged |
| Citraconic anhydride | 16 | 0 | 6 | Unchanged |
| Decitraconylation | 00 | 100 | 100 | - |
| Reductive methylation | 10 | 95 | 100 | - |

**Table II: Chemical modification of the lectin from *A. hirsuta***

| Reagent | Residue modified | No. of residues modified /molecule | Residual Hemagglu -tinating activity % | Buffer used |
|---|---|---|---|---|
| Tetranitromethane | Tyrosine | 4 | 10 | Tris-HCl, pH 8.0 |
| N-acetylimidazole | Tyrosine | 5 | 100 | Phosphate, pH 7.5 |
| N-bromosuccini-mide | Tryptophan | 1 | 100 | Acetate, pH 5.0 |
| Hydroxynitroben-zylbromide | Tryptophan | 0.4 | 100 | Acetate, pH 5.0 |
| Woodward's Reagent K | Carboxyl | 4 | 100 | Phosphate, pH 7.0 |
| Diethyl-pyrocarbonate | Histidine | 4 | 0 | Phosphate, pH 7.0 |
| Photo-oxidation | Histidine | ND | 100 | Phosphate, pH 7.0 |
| Phenylglyoxal | Arginine | ND | 100 | Phosphate, pH 8.0 |
| Phenylmethylsul-phonylfluoride | Serine | ND | 100 | Phosphate, pH 7.0 |

ND: Not Determined

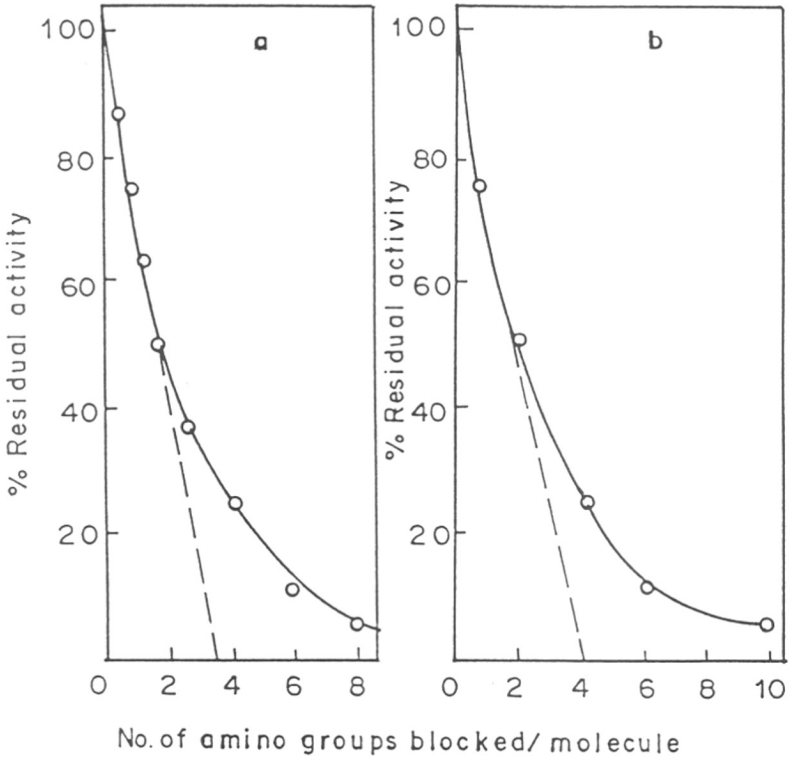


**Fig. III.1:**

**Changes in the activity of *A. hirsuta* lectin as a function of blocked $NH_2$ groups: a) citraconylation b) succinylation**

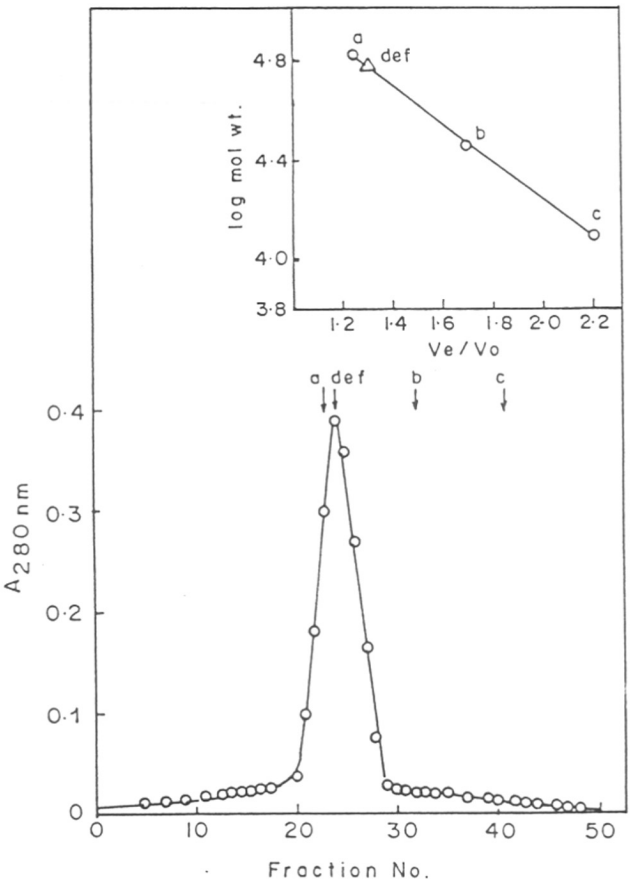


**Fig. III.2:**

**Elution profile of native and modified *A. hirsuta* lectins on gel filtration (Sephadex G-100 column 1.2 cm x 100 cm):** The native, citraconylated and succinylated lectins were found to elute from Sephadex G-100 at the same positions. **Inset** shows the molecular weight plot. The column was equilibrated and operated with phosphate buffer (pH 8.0) containing 0.150 M NaCl and 0.2 M galactose. Calibration was done with (a) bovine serum albumin (b) carbonic anhydrase and (c) cytochrome C. Symbol (d) indicates the native lectin, (e) citraconylated and (f) succinylated lectin.

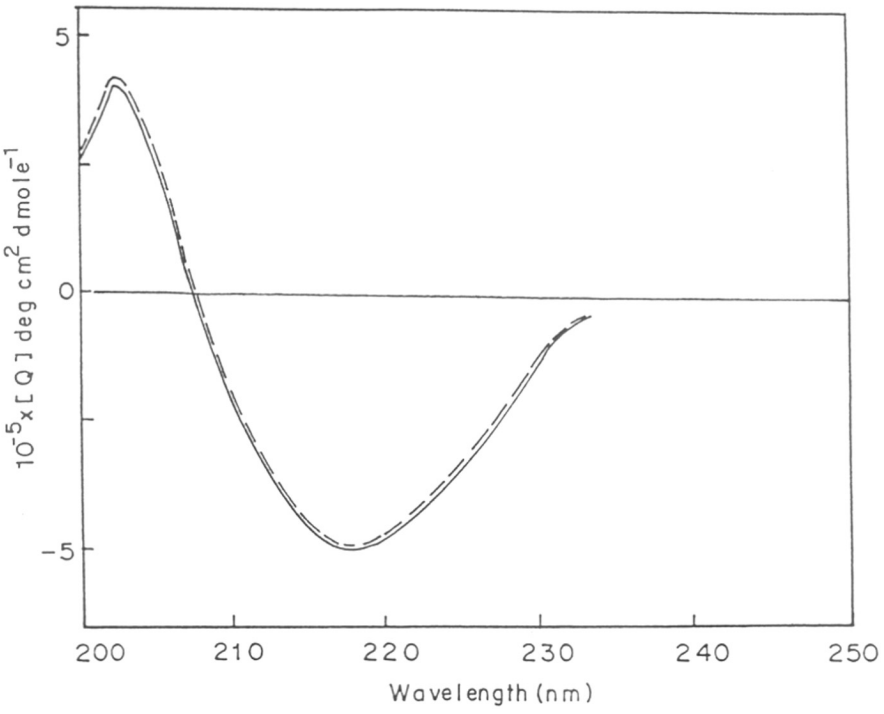


**Fig. III.3:**

**Far UV C.D. spectra of native and chemically modified *A. hirsuta* lectin:** The spectra were recorded on a Jasco-710 spectropolarimeter from 190-250 nm using 1cm pathlength at 25°C at a lectin concentration of 2.5 μM (2 ml) in carbonate buffer, pH 8.5 at a scan speed of 100 nm/min. Native lectin (—) citraconylated lectin (---)

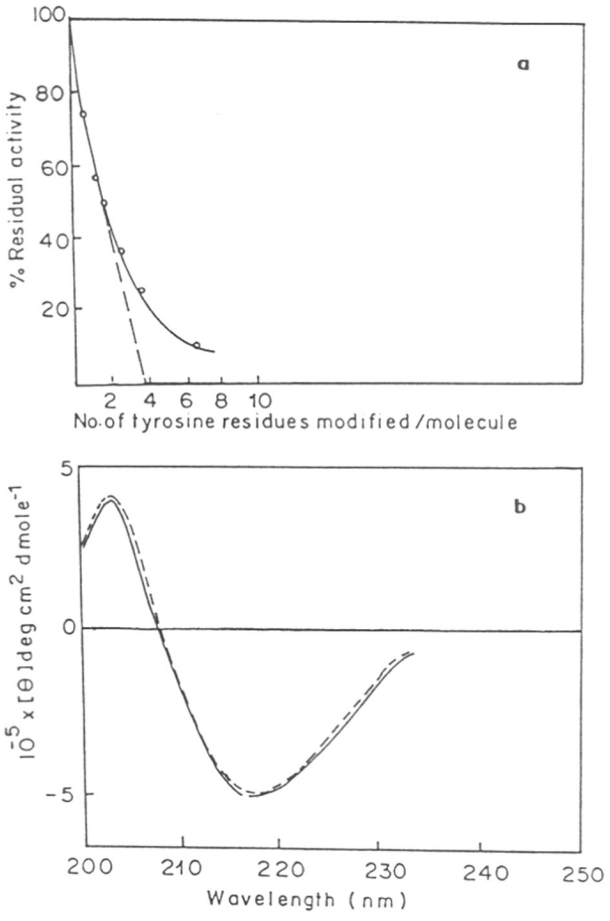


**Fig. III.4:**

**a) Effect of Tetranitromethane on *A. hirsuta* lectin activity:**
Inactivation curve of the TNM modified lectin as a function of modified tyrosine residues

**b) Far UV C.D. spectra of native and TNM modified lectin:**
Spectra of 2.5 μM lectin in Tris-HCl buffer, pH 8.0 was recorded on a Jasco-710 spectropolarimeter. Native lectin (—) and TNM modified lectin (---).

## DISCUSSION

Charged residues like lysine and carboxylate [22,23,24] as well as aromatic amino acids like tryptophan [25-28] and tyrosine [27,29] have been implicated in the sugar binding activity of lectins. Hence modification of these residues were carried out to evaluate their role in the activity of *A. hirsuta* lectin.

Acetylation of *A. hirsuta* lectin with acetic anhydride led to a total loss of its hemagglutination activity suggesting that lysine may have a role in the lectin activity. As acetic anhydride is not specific for lysyl residues but also modifies tyrosine, the role of lysine was evaluated by modifying the lectin with succinic and citraconic anhydrides since these reagents show a preference for lysyl residues [30]. Both succinylation and citraconylation resulted in inactivation of the lectin with concomitant modification of four lysyl residues per molecule of the lectin. The succinic anhydride and citraconic anhydride mediated inactivation could be prevented by pre-incubating the lectin with excess methyl α-galactose. Moreover decitraconylation of the modified lectin restored its initial activity. Furthermore, the C.D. spectra of the succinylated and citraconylated lectins were similar to that of the unmodified lectin suggesting that the loss of activity was due to lysine modification and not as a result of structural changes. The above observations point towards the involvement of lysine in the activity of the *A. hirsuta* lectin.

Conversion of lysine groups to N-monomethyl and N, N'-dimethyl lysine by reductive methylation had no effect on the agglutinating activity. Reductive methylation did not lead to any drastic change in the $pK_a$ of the ε-amino group of lysine nor did it lead to neutralisation/reversal of charge as with acetylation, succinylation and citraconylation; indicating that the

cationic charge of the lysyl residue was essential for the sugar-binding activity of the *A. hirsuta* lectin. Similar effects have been observed in *Ricinus communis* agglutinin and Thaumatin [29,31].

In pea [32], lentil [33] and peanut [34] lectins, lysyl group modification did not affect their ability to bind to the affinity chromatography column although the hemagglutinating activity was lost. However in the *A. hirsuta* lectin, lysine modification resulted in the loss of both sugar-binding as well as hemagglutinating activity suggesting the involvement of lysine residues in both sugar binding and hemagglutination activity. A similar observation has been made in case of the *Ricinus communis* agglutinin [29].

In concanavalin A, lysine modification resulted in the dissociation of the lectin from a tetramer to a dimer [35]. Substitution of the positive charge of amino groups by a negative charge often leads to changes in electrostatic interactions and frequently to dissociation of oligomeric proteins [36]. However, the elution profile of native as well as lysine modified lectin samples on Sephadex G-100 (Fig 1) showed no change, suggesting that modification of lysine residues does not result in the dissociation of the *A. hirsuta* lectin. Similar observations have been reported in case of pea, and lentil lectins as well as peanut and *Ricinus* agglutinins [32-34,29].

Tyrosine has been implicated in the sugar-binding activity of a number of lectins. In Wheat germ agglutinin, NMR results corroborated the crystallographic studies, locating tyrosine in the subsite [37]. In the lentil lectin, the loss of hemagglutinating activity was attributed to the modification of tyrosine by NAI [33]. Nitration of tyrosine of potato lectin [27] and acetylation of *Momordica charantia* and *Ricinus communis* lectins led to a total loss of hemagglutinating activity [38,29].

In the present studies acetylation by NAI did not lead to a concomitant modification of lysine and tyrosine residues of the *A. hirsuta* lectin, instead only tyrosine was modified. Extensive modification of lysine residues by NAI has been reported in the lentil lectin [33] contrary to Con A [39]. In *A. hirsuta* lectin, NAI modified five tyrosine residues with no loss of activity. However, modification of tyrosine with TNM caused the lectin to lose 90 % of its initial activity with concomitant modification of four tyrosine residues. Since NAI acetylates the hydroxyl group whereas TNM nitrates the phenyl ring of tyrosine, the totally opposite effect of the two reagents [40] can be correlated to the involvement of the phenyl ring in the activity of the *A. hirsuta* lectin. The C.D. spectra of the lectin remained unchanged even after modification of the tyrosine residues by TNM, ruling out the possibility of loss in activity due to change in secondary structure. TNM modification of the lectin in the presence of Me α-Gal provided significant protection against loss of activity. The above data indicates the involvement of tyrosine in the activity of the *A. hirsuta* lectin.

Although carbohydrates are highly polar and solvated molecules, sugars have significant non-polar patches formed by the aliphatic protons and carbons at various epimeric centers. Very often this patch is found to be packed against the face of one or more aromatic sidechains of the protein, an arrangement described as stacking in which the galactose ring is parallel to the plane of the aromatic ring [41]. In the case of *A. hirsuta* lectin the phenyl ring of tyrosine may perform the role of a platform for positioning the sugar in the binding pocket, by participating in hydrophobic stacking interactions with the galactopyranose ring similar to tyrosine 248 of the *Ricinus communis* lectin B chain [42].

Carbethoxylation by DEP resulted in total loss of activity. But DEP also acts on cysteine, lysine and tyrosine. Hence only reversal of

inactivation by hydroxylamine hydrochloride can confirm the involvement of histidine in the saccharide-binding activity. Attempts to reverse the DEP mediated inactivation using various concentrations of hydroxylamine, at various pH values and different time intervals proved futile, suggesting that the inactivation may be due to the modification of other residues. Involvement of cysteine was ruled out due to its absence in the *A. hirsuta* lectin. Moreover, there was no decrease in the absorbance at 278 nm suggesting that DEP mediated inactivation is not due to tyrosine modification. However, the estimation of amino groups with TNBS following DEP treatment indicated the modification of lysine residues. The failure of hydroxylamine to reactivate a DEP modified protein has been ascribed to modification of lysine residues [21]. Furthermore, reversible blocking of the amino groups by citraconic anhydride prior to carbethoxylation resulted in no loss of activity, confirming that histidine has no role in the saccharide binding activity. Lack of lectin inactivation by photo-oxidation further confirmed the absence of histidine in the binding site.

Contrary to the observations made in case of wheat germ agglutinin [25], *Abrus precatorius* seed lectin [26], potato (*Solanum tuberosum*) lectin [27] and *Pisum sativum* lectin [28], the modification of tryptophan residues in *A. hirsuta* lectin did not bring about any loss of activity, indicating that it has no role in the activity of the lectin. Chemical modification studies also revealed that serine, arginine and carboxylate residues are not involved in the saccharide binding activity of *A. hirsuta* lectin.

In conclusion, the phenyl ring of tyrosine and the positive charge of the ε-amino group of lysine are essential for activity of the *A. hirsuta* lectin. Together these residues may be involved in the docking and bonding of sugars to the lectin.

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# CHAPTER IV

# FLUORIMETRIC STUDIES ON *ARTOCARPUS HIRSUTA* LECTIN

# SUMMARY

The intrinsic fluorescence intensity of *Artocarpus hirsuta* lectin was enhanced upon binding to galactose derivatives, without any change in the emission maximum (333 nm). The enhancement observed with methyl α-galactose was 55%. The association constant of methyl β-galactose is lower than that of galactose, which can be attributed to the equatorial orientation of the C-1 methyl group. The association constants for these sugars, decreased with increase in temperature. The enthalpy values calculated for methyl α-galactose, galactose and methyl β-galactose from the van't Hoff plots were similar and in the range -50 to -55 $kJmol^{-1}$. The difference in affinities therefore reflected the difference in the entropy. ΔS values for methyl α-galactose, galactose and methyl β-galactose were -85 $Jmol^{-1}K^{-1}$, -111 $Jmol^{-1}K^{-1}$ and -150 $Jmol^{-1}K^{-1}$ respectively. Methylumbelliferyl α-galactose showed the highest affinity and a relatively positive entropic value of -13.2 $Jmol^{-1}K^{-1}$. The increased affinities of methylumbelliferyl galactose and methyl α-galactose over galactose could be attributed to apolar interactions of the methylumbelliferyl group and methyl group with a hydrophobic pocket at/near the sugar binding site.

Solute quenching studies of the lectin using acrylamide, potassium iodide and cesium chloride were carried out in the absence and presence of methyl α-galactose. These studies showed that one of the four tryptophan residues in the lectin, is present on the surface and is accessible to the ionic quenchers. This tryptophan could be in the vicinity of positively charged amino acid residues, near the sugar-binding site and could contribute to the enhancement in the fluorescence of the lectin on sugar binding. The other three tryptophans are probably in a hydrophobic environment and accessible only to the neutral quencher.

# INTRODUCTION

Cell recognition is a key event in biological processes like fertilization, embryogenesis and defence [1]. Lectins have been implicated in such processes by virtue of their specificity of interaction with carbohydrate receptors. Biological activities of lectins are a manifestation of their carbohydrate specificities.

It is essential to understand the mechanism of ligand binding to lectins, in-order to facilitate their use as an analytical tool and for better understanding of lectin interaction with cell bound carbohydrates. Determination of association constants with a series of ligands provides considerable insight into the spatial features of a lectin's combining site. Complementary thermodynamic data offers information on the forces involved in the binding and explains affinity differences encountered.

Since hemagglutination-inhibition studies provide semi-quantitative information, fluorescence spectroscopy has been used to obtain quantitative information about a lectin's binding process. The advantage of using fluorimetry in studies of carbohydrate–protein interaction is that the binding can be studied at equilibrium without physical separation of the bound complex from the free ligand and the protein [2]. Fluorescence of tryptophan is influenced by its microenvironment; hence changes which affect the tryptophan environment can cause changes in fluorescence properties [3].

Quenching studies provide valuable information about the exposure of protein bound intrinsic/extrinsic fluorophores [4]. Details of the microenvironment of the tryptophan residues and its accessibility can be obtained by comparing the quenching profiles for a protein using different quenchers.

Thermodynamic parameters for the binding of a variety of saccharides to *A. integrifolia* lectin have been investigated and found to be most favourable for the binding of Gal β1-3 GalNAc to the lectin, suggesting an extended binding site. The major stabilising force was found to be enthalpic [5]. In comparison, for the *A. lakoocha* lectin, only the binding constants for its interaction with 4-methylumbelliferyl α-galactoside have been determined [6].

In this chapter, determination of the binding constants for the association of ligands with the *A. hirsuta* lectin, by ligand-induced enhancement of protein fluorescence are reported. Thermodynamic parameters for saccharide binding have also been determined and the accessibility of tryptophan residues of the lectin has been studied.

## MATERIALS AND METHODS

### *Materials*

Galactose, galactosamine, 2-deoxygalactose, L-fucose, glucose, mannose, methyl α-glucose, methyl α-mannose, methyl α-galactose, melibiose, Gal β 1-3 GalNAc, 4-methylumbelliferyl α-galactose, 4-methylumbelliferyl β-galactose, and other sugars as well as cesium chloride and acrylamide were purchased from the Sigma Chemical company (St. Louis, U.S.A.). Potassium iodide used was from S.D. Fine Chemicals. All other chemicals were of analytical grade.

## *Methods*

### Purification of *Artocarpus hirsuta* basic lectin

The lectin was purified from seeds as described in chapter II.

### Determination of concentration

Protein concentration was determined by the method of Lowry et al. using bovine serum albumin as standard [7]. The concentration of 4-methyl umbelliferyl α-galactose (MeUmb α-Gal) was determined spectrophotometrically from its molar absorbance of $1.36 \times 10^4$ $M^{-1}cm^{-1}$ at 318 nm [8]. All experiments were carried out in Tris HCl buffer pH 7.2, containing 150 mM NaCl (THS) and solutions were filtered through 0.45 μm filter before use.

### Fluorescence measurements

Fluorescence measurements were made using a Perkin Elmer LS 5B Spectrofluorimeter, with slit width of 5 nm for both the monochromators. Samples were placed in a quartz cuvette maintained at constant temperature (± 0.1°C) by means of a circulating cryobath. Samples were excited at 280 nm and the emission was monitored at 333 nm ($\lambda_{max}$ of the lectin) Corrections were also made to compensate the dilution effect upon addition of sugar/lectin. At the highest concentration of the saccharide/lectin, volume change was less than 5 % of the solution in the cuvette.

The binding of non-fluorescent sugars was studied by intrinsic fluorescence titrations. To 2 ml lectin sample ($A_{280}$ 0.1) in THS, 5-60 μl aliquots of the non-fluorescent sugar solution were added and the fluorescence intensity monitored at 333 nm before and after addition. From the ordinate intercept of the double reciprocal plot of $F_0 / F_c - F_0$ versus 1/[C], where $F_0$ and $F_c$ are the fluorescence intensities of the free protein and of

the protein at a saccharide concentration [C], $F_\infty$ the fluorescence intensity at infinite sugar concentration was obtained. In the plot of log [C] versus log $[F_c-F_0 / F_\infty-F_c]$, the abscissa intercept yielded the $K_a$ value for the lectin-sugar interactions. The association constants were determined from the titration data [9] by assuming the relation that $pK_a$ of the complex equals the value of [C] when $\log (F_c-F_0/F_\infty-F_c) = 0$

For the fluorescent sugar, a fixed volume (2 ml) of 4-methylumbelliferyl α-galactose (MeUmb α-Gal) (2.2 μM) was taken in the cuvette and titrated by addition of 5-60 μl aliquots of *A. hirsuta* lectin (166 μM). The fluorescent sugar was excited at 318 nm and spectra recorded above 330 nm both in the absence and after addition of each aliquot of the lectin. A double reciprocal plot of fluorescence intensity $F_0/F_0-F_c$ versus protein concentration 1/[P] gave the fluorescence intensity at infinite protein concentration $F_\infty$, where $F_0$ and $F_c$ are the fluorescence intensities of the fluorescent sugar alone and of the fluorescent sugar at a lectin concentration [P]. In the plot of log [P] against log $[F_0-F_c / F_c-F_\infty]$, the abscissa intercept yielded the $K_a$ value for the interaction of MeUmb α-Gal with *A. hirsuta* lectin.

Free energy changes of association (ΔG) were determined by the equation; $\Delta G = - RT \ln K_a$ (1)

Temperature dependence of the association constants was made use of to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the van't Hoff plots by using the equation;

$$\ln K_a = (-\Delta H/RT) + \Delta S/R \quad (2)$$

The slope of the plot equals $(-\Delta H/R)$ from which the enthalpy change was calculated. The entropy change was obtained from the equation;

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

***Solute Quenching*:** Titrations with acrylamide, CsCl and KI were performed in the presence and absence of 0.1 M Me α-Gal. The iodide solution contained sodium thiosulphate (200 μM) to suppress tri-iodide formation. Defined amounts of the quencher (5μl) were added from a stock of 5.0 M to a 2 ml protein solution ($A_{280}$ 0.1) in the cuvette. All quenching experiments were done in THS. The fluorescence intensity was recorded after 4 minutes and data was analysed according to the following equations [10,11].

$$F_0 / F_0\text{-}F = 1 / f_a + 1/ f_a K_q [Q] \quad (4)$$

$$F_0 / F = 1 + K_q [Q] \quad (5)$$

F and $F_0$ are the fluorescence intensities at 333 nm in the presence and absence of the quencher at concentration [Q] respectively.

## RESULTS

Addition of galactose derivatives resulted in enhancement of the lectin fluorescence with no shift in the emission maximum. The maximum enhancement in the intrinsic fluorescence of the lectin, on binding with methyl α-galactose (Me α-Gal) was 55% (Fig. IV.1). Methyl β-galactose (Me β-Gal) was the least complementary sugar (Fig. IV.2). The slope of the plot of log $F_c$-$F_0$/ $F_\infty$-$F_c$ versus log [C] was unity for all the sugars used. The association constants obtained for the binding of various sugars at 27°C are listed in Table I.

The fluorescence intensity of 4-methylumbelliferyl α-galactose, was quenched 100 % on titration with the lectin, while for the same concentration of protein and fluorescent sugar, the fluorescence intensity of 4-methylumbelliferyl β-galactose remained unchanged. The values of

association constants determined at different temperatures for Me α-Gal, Gal, Me β-Gal and MeUmb α-Gal are listed in Table II. The association constants decreased with increase in temperature corresponding to a negative change in enthalpy (ΔH), (Fig. IV.3). The increase in the affinity of the Me α-Gal and MeUmb α-Gal over galactose was accompanied by a positive entropy change (Table III).

Titration of the lectin with acrylamide, KI and CsCl resulted in 100 %, 28 % and 18 % quenching of the fluorescence of the native protein, respectively. The modified Stern Volmer plots ($F_0/F_0$-F versus 1/[Q]) for all three quenchers were linear (Fig. IV.4A) while the direct Stern Volmer plot ($F_0/F$ versus [Q]) showed upward curvature for acrylamide, downward for KI and was straight for CsCl (Fig. IV.4B). In the modified Stern Volmer plots, $f_a^{-1}$ was given by the intercept. Accordingly, the fraction of tryptophan residues accessible to quenchers ($f_a$) in the free lectin and its complex with lactose were expressed in values relative to the tryptophan residues contributing to the initial fluorescence of the lectin (Table IV).

**Table I: Association constants for *A. hirsuta* lectin-sugar interactions**

| Monosaccharides | $K_a$ ($M^{-1}$) at 27°C | - $\Delta G$ ( kJ $M^{-1}$) |
|---|---|---|
| Galactose | 758 | 16.51 |
| Methyl α-galactose | 20,000 | 24.65 |
| Methyl β-galactose | 90 | 11.2 |
| 2-deoxygalactose | 708 | 16.33 |
| Galactosamine | 769 | 16.56 |

| Disaccharides | $K_a$ ($M^{-1}$) at 27°C | - $\Delta G$ ( kJ $M^{-1}$) |
|---|---|---|
| Gal α1-3 Gal | 550 | 15.69 |
| Gal α1-4 Gal | 224 | 13.7 |
| Gal α1-6 Glc | 1000 | 17.18 |
| Gal β1-3 GalNAc | NB | — |
| Gal β1-3 GlcNAc | NB | — |
| Gal β1-4 Glc | NB | — |

| Trisaccharides | $K_a$ ($M^{-1}$) at 27°C |
|---|---|
| Gal α1-3 Gal β1-4 GlcNAc | NB |
| Gal α 1-3 Gal β1-4 Gal | NB |
| Gal α (Fuc α1-2) Gal | NB |

NB stands for non-binding

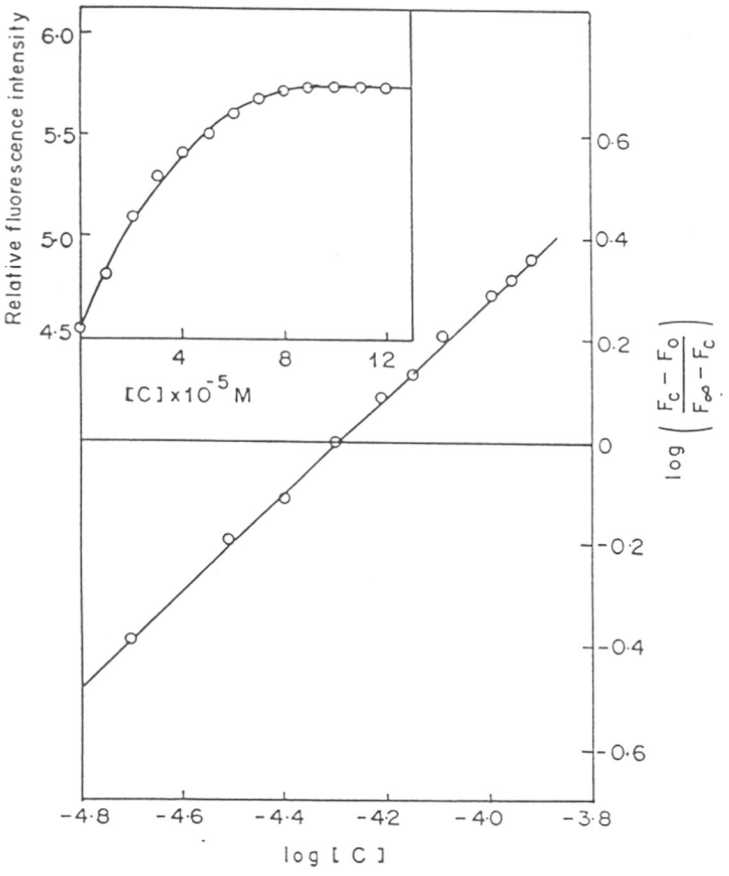


**Fig. IV.1:**

**Evaluation of the association constant for the binding of methyl α-galactose to *A. hirsuta* lectin by intrinsic fluorescence at 27°C:** Plot for the determination of the association constant. **Inset:** Enhancement of the intrinsic fluorescence of *A. hirsuta* lectin upon its titration with Me α-Gal.

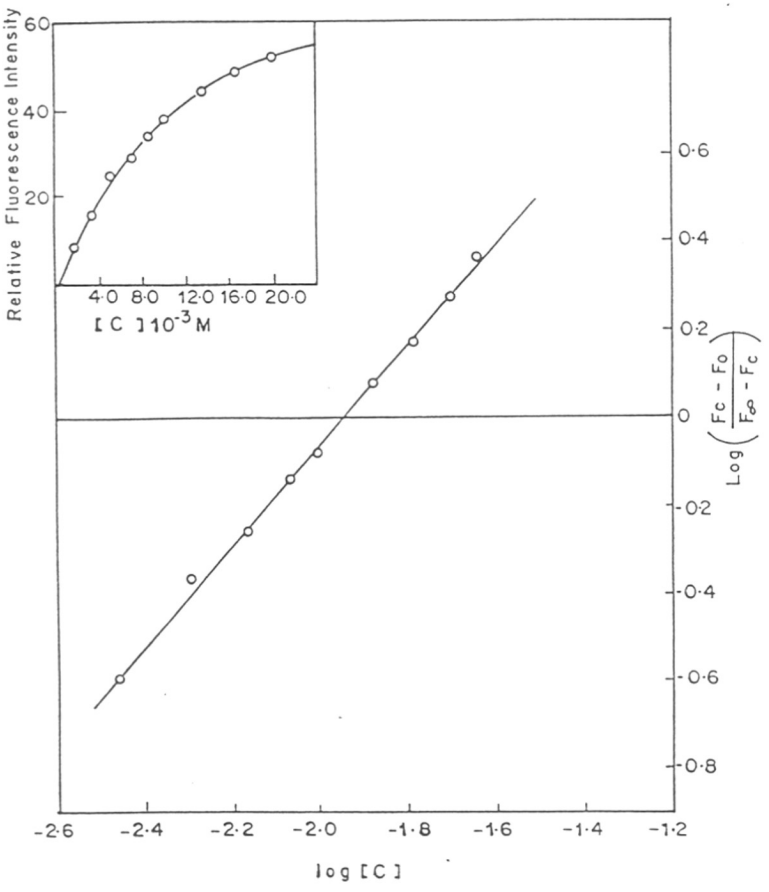


**Fig IV.2:**

**Evaluation of the association constant for the binding of methyl β-galactose to *A. hirsuta* lectin by intrinsic fluorescence at 27°C:** Plot for the determination of the association constant. **Inset:** Enhancement of the intrinsic fluorescence of *A. hirsuta* lectin upon its titration with Me β-Gal.

**Table II : Association constants for the binding of *A. hirsuta* lectin to galactose and its derivatives.**

| Sugar | $10^{-3} \times K_a$ ($M^{-1}$) | | | | |
|---|---|---|---|---|---|
| | 15°C | 20°C | 25° C | 30°C | 35°C |
| Me α-Gal | 45.7 | 36.3 | 25.1 | 16.6 | — |
| Gal | 1.7 | 1.45 | 1.0 | 0.65 | — |
| Me β-Gal | — | 0.15 | 0.1 | 0.07 | 0.05 |
| MeUmb α-Gal | 550 | 457 | 330 | 276 | 191 |

**Table III: Thermodynamic parameters for the binding of *A. hirsuta* lectin to galactose and its derivatives.**

| Sugar | $10^{-3} \times K_a$* $M^{-1}$ | - ΔH kJ.mol-1 | - ΔG* kJ.$mol^{-1}$ | - ΔS J.$mol^{-1}.K^{-1}$ |
|---|---|---|---|---|
| Me α-Gal | 36.3 | 50.46 | 25.17 | 85.5 |
| Gal | 1.45 | 49.88 | 17.22 | 111 |
| Me β-Gal | 0.15 | 56.20 | 11.02 | 150 |
| MeUmb α-Gal | 457 | 35.54 | 31.46 | 13.2 |

* Values were calculated at 20°C.

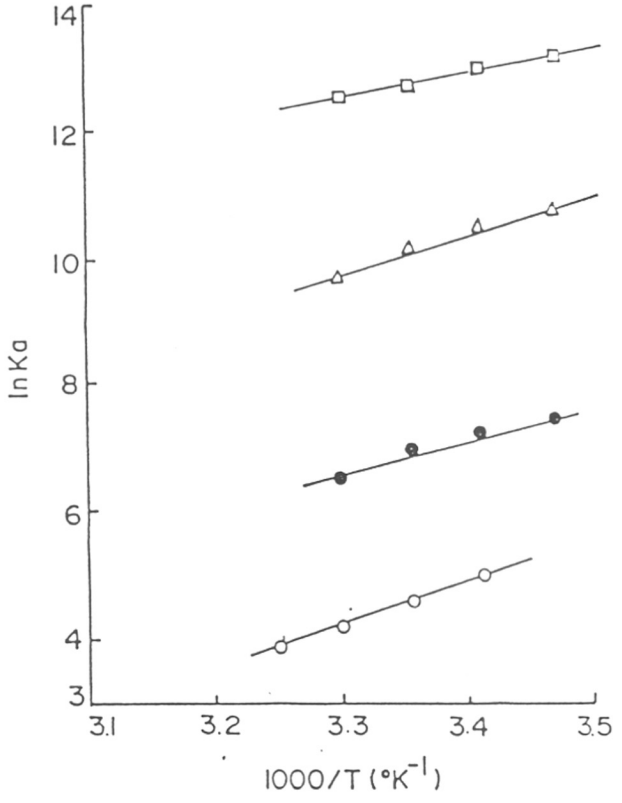


**Fig. IV.3:**

**Van't Hoff plots for the association of sugars to *A. hirsuta* lectin:**
The symbols used are; methylumbelliferyl α-galactose (□), methyl α-galactose (Δ), galactose (●) and methyl β-galactose (O). The association constants were determined at 15°C, 20°C, 25°C, 30°C and 35°C

**Table IV: Quenching data on *Artocarpus hirsuta* lectin fluorescence by solute quenchers.**

| Quencher | Protein | $f_a$ | mol tryptophan accessible per mol of lectin |
|---|---|---|---|
| Acrylamide | Lectin | 1.04 | 4 |
| | Lectin in 0.1M Me α-Gal | 1.04 | 4 |
| KI | Lectin | 0.18 | 0.7 (1) |
| | Lectin in 0.1M Me α-Gal | 0.18 | 0.7 (1) |
| CsCl | Lectin | 0.185 | 0.7 (1) |
| | Lectin in 0.1M Me α-Gal | 0.185 | 0.7 (1) |

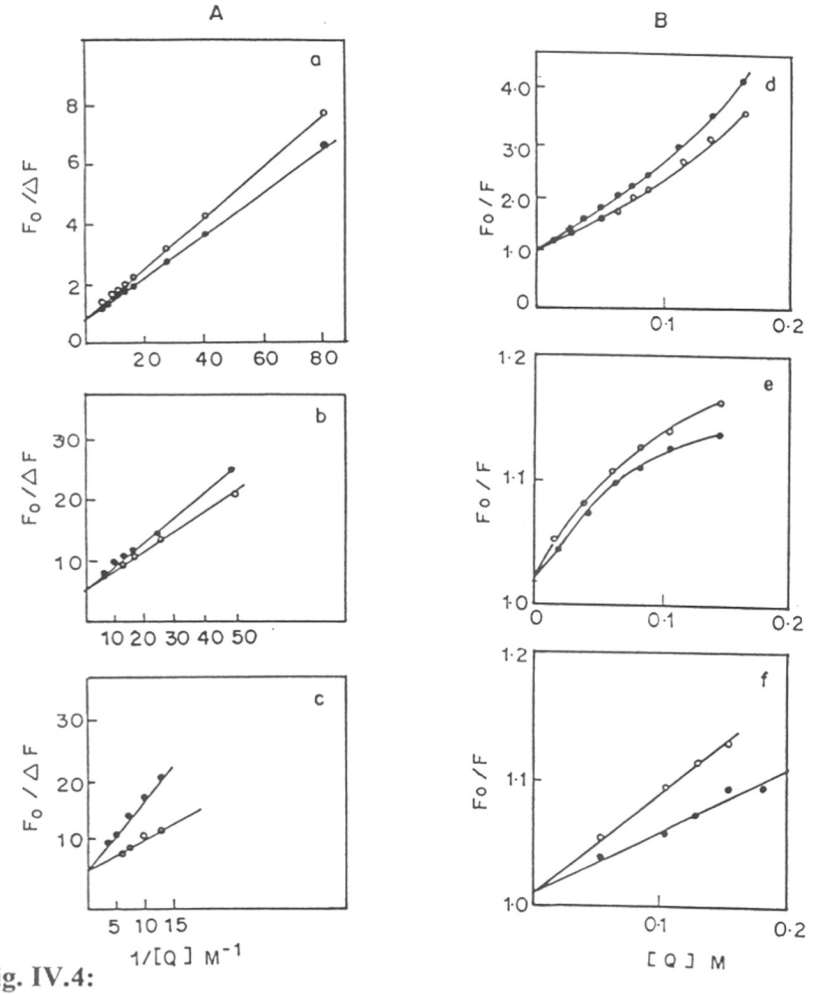


**Fig. IV.4:**

**Stern Volmer and modified Stern Volmer plots for fluorescence quenching of the *A. hirsuta* lectin upon addition of solute quenchers:** The fraction of tryptophan residues exposed was calculated from the ordinate intercept. Solute quencher stock concentrations were 5M. The symbols used are (O) in the absence of sugar and (●) in the presence of sugar.

**A] Modified Stern Volmer plots ; a)** Acrylamide **b)** KI **c)** CsCl
**B] Direct Stern Volmer plots ;** **d)** Acrylamide **e)** KI **f)** CsCl

## DISCUSSION

The near UV C.D. spectrum of the lectin was altered by sugars with no change in the far UV C.D. spectrum, indicating that the sugars did not cause any conformational change in the polypeptide backbone of the lectin (Chapter II). The binding equilibrium of the lectin with sugars could therefore be described by a single step association reaction v.i.z. P + L = PL; $K_a$ = [PL]/ [P][L] [12]. The association constants ($K_a$) could therefore be determined by the method of Chipman [9].

The change in fluorescence intensity of the lectin, as a function of saccharide concentration has been utilised to determine the association constants. The primary specificity of the lectin is directed towards galactose and the affinities of galactose derivatives are in good agreement with those obtained by hemagglutination inhibition studies with rabbit erythrocytes (Chapter II). There is no change in the fluorescence intensity of the lectin on titration with glucose, mannose, their methyl or amine derivatives as well as with fucose, indicating that these sugars do not bind and that the C-4 and C-6 hydroxyl groups are important binding loci. The affinity constants for 2-deoxy galactose (708 $M^{-1}$) and galactosamine (769 $M^{-1}$) are similar to galactose (758 $M^{-1}$) indicating that the C-2 position is not critical for binding. Me α-Gal is the most active ligand; its association constant for the *A. hirsuta* lectin (20,000 $M^{-1}$) is 26 fold higher than that of galactose and 222 fold higher than that of Me β-Gal (90 $M^{-1}$) indicating a favourable and strong association between Me α-Gal and the lectin due to the axial methyl group. The same group in the β position has a destabilising effect due to its equatorial position. However the presence of a methyl group at C-1 position in the α-anomer alone is not sufficient to cause effective binding since Me α-Glc and Me α-Man fail to bind to the lectin. The preference for

the α anomer over the β anomer of galactose has also been observed for WBA II [13] and *Griffonia simplicifolia* $B_4$ lectin [14].

The α-linked disaccharides Gal α1-3 Gal, Gal α1-4 Gal and Gal α1-6 Glc (melibiose) did not induce the same change in fluorescence intensity of the lectin, as with Me α-Gal, nevertheless there was binding with lesser affinity. This is indicative of the fact that in α-linked disaccharides, the replacement of the 1-0-methyl group in galactose by a hexapyranose residue could lead to unfavourable projection of hydrophilic hydroxyl groups in the binding site of the lectin accounting for their $K_a$ values being less than that of Me α-Gal. The $K_a$ for Gal α1-3 Gal (550 $M^{-1}$) is greater than that of Gal α1-4 Gal (224 $M^{-1}$) as the reducing sugar is projected away from the non-reducing sugar in the former and there is no hindrance to the terminal galactose [15]. The affinity for Gal α1-6 Glc (melibiose 1000 $M^{-1}$) is even better than the other two disaccharides as there is greater flexibility for rotation about the α1-6 linkage and this could lead to its increased affinity [16]. Gal α1-6 Glc by virtue of having a single galactopyranose residue is expected to bind through its non-reducing sugar alone. Thus it appears that the *A. hirsuta* lectin interacts with these sugars through their non-reducing hexapyranose residue.

There is no binding to β linked disaccharides Gal β1-3 GalNAc, Gal β1-3 GlcNAc and lactose (Gal β1-4 Glc) which underlines the specificity for α-galactose. Inspite of strong affinity for Me α-Gal the lectin does not bind the B blood group determinant trisaccharides [Gal α1-3 (Fuc α1-2) Gal, Gal α1-3 Gal β1-4 Gal, Gal α1-3 Gal β1-4 GlcNAc]. Results indicate the *A. hirsuta* lectin possibly does not have an extended binding site unlike the *A.integrifolia* lectin, which shows highest affinity for disaccharides and is proposed to have two subsites.

Enthalpy values for Gal, Meα-Gal and Me β-Gal are almost constant (-49.88 to -56.2 $kJmol^{-1}$) indicating the contribution of binding enthalpy to the differences in the free energy of binding for these sugars is not significant. The higher or lower affinities therefore are a manifestation of whether the contribution of entropy is favourable or not. On comparison of ΔS values of galactose (-111 $Jmol^{-1}K^{-1}$) and Me α-Gal (-85.5 $Jmol^{-1}K^{-1}$) it appears that the methyl group at C-1 is favourable but the highly negative ΔS value of Me β-Gal (-150 $Jmol^{-1}K^{-1}$) suggests that the presence of the same group in β linkage to galactose is detrimental for the binding process.

There is considerable influence of the umbelliferyl group on the binding parameters of 4-methylumbelliferyl α-galactose to the lectin, as $K_a$ ($3.3 \times 10^5$ $M^{-1}$) increases 17 fold relative to Me α-Gal. The origin of this effect is largely entropic and the value of ΔS (-13.2 $Jmol^{-1}K^{-1}$) is relatively positive compared to ΔS values obtained for other galactose derivatives studied. A relatively positive ΔS indicates a non-polar site at/adjacent to the galactose binding site as well as enhanced non-polar interactions between the aglycone and the combining site of the lectin. This may be due to the release of water molecules during the binding process [17]. Another observation is the decrease in the exothermic character of the binding process. This decrease is compensated for by the larger entropic effect resulting in an increased affinity for the lectin. In contrast to the 100 % quenching of the fluorescence intensity of 4-methyl umbelliferyl α-galactopyranoside, the fluorescence intensity of 4-methyl umbelliferyl β-galactopyranoside is unaltered, indicating that the sugar does not bind to the lectin.

Buried tryptophan residues in protein show emission maxima in the range 325-335 nm [18]. *Artocarpus hirsuta* lectin shows an emission maximum of 333 nm irrespective of excitation at 280 or 295 nm indicating

the presence of tryptophan residues in a hydrophobic environment. Although tryptophan fluorescence is sometimes interpreted as 'involvement of tryptophan in binding' it may not be the consequence of direct participation of tryptophan in saccharide-binding [2]. Our chemical modification studies reveal the absence of tryptophan in the saccharide binding site (Chapter III). Since fluorescence of the lectin is enhanced on ligand binding, microenvironment of tryptophan was studied using solute quenchers.

The upward curvature of acrylamide (direct Stern Volmer plot) indicates the occurrence of both static and dynamic quenching. Low quenching by KI (28%) and CsCl (18%) in contrast to 100 % quenching by acrylamide suggests that the tryptophans are not accessible to ionic quenchers. This low accessibility is consistent with the $\lambda_{max}$ of 333 nm observed for the lectin, indicating that the tryptophans are in a hydrophobic environment. Among the ionic quenchers higher efficiency of quenching by KI as compared to CsCl suggests an electropositive microenvironment around tryptophan. This observation is in agreement with our studies on chemical modification of the lectin, which shows the presence of positively charged ε-amino groups at the sugar-binding site (Chapter III).

Since ionic quenchers do not permeate proteins, it is possible to determine fraction of total fluorescence due to surface tryptophans. Our previous data (Chapter II) reveals four tryptophans are present in the lectin molecule. The fraction of $Cs^+$ accessible tryptophan (0.18 ie 1 tryptophan) and $I^-$ accessible tryptophan (0.18 ie 1 tryptophan) were less compared to acrylamide (1.04 ie 4 tryptophans). $f_a$ in the presence and absence of saccharide was the same indicating no change in the number of tryptophans accessed upon ligand binding. In *Abrus precatorius* agglutinin galactose binding decreased the accessibility of these residues towards iodide [19] while in *Momordica charantia* lectin lactose increased the accessibility of

tryptophans towards iodide [20]. Changes in the conformation of the protein due to increasing quencher concentrations may affect fluorescence parameters but in all cases there is no spectral shift or loss of activity.

Fluorescence studies for the *A. hirsuta* basic lectin interaction with sugars indicate that it is a spontaneous, exothermic binding process. The major driving force for the avid binding of Me α-Gal and MeUmb α-Gal as compared to galactose appears to be entropic. One of the four tryptophan residues in the lectin is present on the surface and is accessible to the ionic quenchers. This tryptophan could be in the vicinity of positively charged amino acid residues, near the sugar-binding site and could contribute to the enhancement in the fluorescence of the lectin on sugar binding. The other three tryptophans are probably in a hydrophobic environment and accessible only to the neutral quencher.

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# CHAPTER V

# GENERAL DISCUSSION AND CONCLUSIONS

## DISCUSSION

Lectins constitute a class of proteins or glycoproteins having selective binding affinity for carbohydrates, glycoconjugates and polysaccharides. They occur in a wide variety of microorganisms, plants and animals. A large number of these lectins have been purified and characterised in detail [1, 2]. Due to their high degree of specificity, they have often been employed as highly discriminating agents in studies of normal and malignant cells, in blood typing, purification of polysaccharides and glycoproteins, in the fractionation of cell populations and in the investigation of carbohydrate structure.

Based on their sugar specificity, lectins can be classified into specificity groups namely, galactose/ their derivatives, mannose/glucose/their derivatives, sialic acid and fucose binding lectins. In plants, majority of the studies have been concentrated on the first two sugar specificity groups and especially on lectins from the Leguminoceae family. Lectins belonging to the family Moraceae are comparatively less studied. The Moraceae family includes trees and shrubs with milky sap and unisexual flowers. *Ficus, Morus, Streblus, Artocarpus* are some genera from this family. There are forty species of the *Artocarpus* genus found in the region stretching from the Indian subcontinent upto the Pacific Ocean. Lectins from six species of *Artocarpus* have been investigated, namely *A. integrifolia, A. lakoocha, A. champeden, A. tonkinensis, A. incisa* and *A. altilis* [3-8]. The lectin from *A. hirsuta,* a species found south of Konkan, in India, has been described in this thesis. The purpose of this discussion is to elucidate the position of the *Artocarpus hirsuta* lectin relative to other *Artocarpus* lectins and plant lectins.

*A. hirsuta* lectin has been purified by sequential ion-exchange chromatography as well as by affinity chromatography on cross-linked guar gum. Cross-linked guar gum has been used for the isolation of galactopyranoside binding lectins and in some cases N-acetylgalactosamine binding agglutinins, like *Bandeiraea simplicifolia*, *Ricinus communis*, soybean and wild cucumber lectins [9]. Lectins from other *Artocarpus* species too have been purified both by conventional techniques such as ion-exchange chromatography [10] and by affinity binding on, IgA-Sepharose [11], Affigel Galactosamine-Agarose [12], desialyted Mucin-Sepharose-4B [5], Rivanol [4], cross-linked guar gum [3], Melibiose-Agarose [13] and Mannose-Sepharose [14].

The molecular mass of *Artocarpus hirsuta* lectin is 60 kDa with subunits of 14.2 and 15.8 kDa. The molecular mass of lectins from *A. integrifolia* and *A. lakoocha* range between 39 to 70 kDa [4,15,16]. The *Artocarpus* lectins are $\alpha_2\beta_2$ type heterotetramers with non-covalently associated subunits. The molecular mass of the $\alpha$ subunit in *Artocarpus* lectins range between 15 to 18 kDa and that of $\beta$ subunit between 11 to 14 kDa. The mannose binding lectin of *A. integrifolia* (artocarpin) is a homotetramer [14]. The galactose binding lectin of *A. integrifolia* (jacalin) has also been reported to be a homotetramer [16,13].

*A. hirsuta* lectin has a pI of 8.5. The presence of isolectins with pI values between 5.0-8.0 have been observed in jacalin [17,18], between pH 8.8 and 9.0 in *A. incisa* lectin [7] and between 5-6.5 in the *A. integrifolia* mannose specific lectin (artocarpin) [14]. Isolectins have been reported in a number of seed lectins and sometimes these consist of subunits with different sugar specificities or different biological properties [19,20]. The complexity observed in jacalin is higher than that observed with other plant lectins but the isolectins do not show any difference in carbohydrate specificity [21].

With the exception of artocarpin, other *Artocarpus* lectins are glycoproteins. The *A. hirsuta* lectin has 6 % neutral sugar content. The *A. integrifolia* lectin has 3-7 % carbohydrate content [3,17] and the carbohydrate moiety consists of xylose (4.1%) mannose (0.9%), galactose (1.1%), galactosamine (1.6 %) and trace amounts of glucose and mannosamine [17]. The lectin from *A. lakoocha* has 11.7 % carbohydrate with 6 % xylose as the main sugar [13]. *A. incisa* lectin has 2.1% carbohydrate content [7]. Ruffet et al. [22] have shown that the major sugar in jacalin is N-linked.

The amino acid composition of the lectins from *A. integrifolia* [18], *A. champeden* [5], *A. tonkinensis* [6], *A. lakoocha* [13], *A. incisa* [7], *A. altilis* [8] and *A. hirsuta* are fairly similar. However, the lectin from *A. lakoocha* differs from lectins of other *Artocarpus* species in that, it has no proline, almost half the amount of glycine, valine and a high amount of tryptophan.

The *A. hirsuta* lectin like many other purified lectins is a metalloprotein and its activity like those of the lectins from *Datura stramonium, Maclura pomifera* and *Artocarpus integrifolia* is not enhanced by the addition of metal ions [23,24,17]. Lectins from *Canavalia ensiformis,* soybean, *Dolichos biflorus* require $Mn^{2+}$ and/or $Ca^{2+}$ for activity [19,25,26]. Atomic absorption spectroscopy showed the presence of $Ca^{++}$ in *A. hirsuta* lectin and $Fe^{++}$, $Ca^{++}$, $Mg^{++}$, $Cr^{+++}$, $Zn^{++}$ and $Mn^{++}$ in *A. integrifolia* lectin [17]. *A. lakoocha* lectin is also a metalloprotein [4].

*A. hirsuta* lectin showed a high hemagglutination titre and agglutinated erythrocytes without any prior proteolytic treatment unlike certain plant lectins (*Arachis hypogea*) which require prior treatment of the erythrocytes with neuraminidase, pronase or trypsin. The lectins from *A. lakoocha, A. integrifolia* and *A. hirsuta* agglutinate human erythrocytes of

all blood groups [4,17]. In addition *Artocarpus integrifolia* lectin agglutinates erythrocytes from mouse, duck, pigeon and buffalo [17].

*A. hirsuta* lectin remained stable over a wide pH range, in contrast to the peanut agglutinin tetramers which dissociate into dimers [27] and wheat germ agglutinin (WGA) dimers which dissociate into monomers [28] at low pH values.

Information regarding the sugar specificity of lectins is based on studies by inhibition of hemagglutination, inhibition of precipitin reaction as well as by fluorescence spectroscopy. The α-anomer of galactose is preferred by the *A. hirsuta, Maclura pomifera* [29] *Griffonia simplicifolia* [19] and winged bean agglutinins (WBA) [30] while the β-anomer of galactose is preferred by the snake gourd [31] and *Momordica charantia* lectins [32]. In galactose specific lectins like *Ricinus communis* and *A. hirsuta* the preference for the α/β anomer is retained on going from the 'alkyl' to the 'aromatic' aglycone [33] while lack of distinction between α and β anomers of 'aromatic' glycosides has been observed in *G. simplicifolia* lectin [19]

Jacalin, *A. lakoocha* and *A. hirsuta* lectins bind galactose derivatives while glucose, mannose or their derivatives are not inhibitors of lectin activity, indicating the importance of the axial C-4 hydroxyl [34,35]. The specificity shown by α–anomers of galactose derivatives may be due to the fact that the hydroxyl at C-1 being axial in them is accessible to the lectin-binding site while that in the β-anomer, being equatorial in position may sterically hinder lectin carbohydrate interaction. Me α-Gal is the common effective inhibitor of lectin activity in all the three *Artocarpus* species. *A. hirsuta* lectin binds Me α-Gal 222 fold stronger than Me β-Gal similar to jacalin [34] whereas *B. simplicifolia* lectin I $B_4$ and peanut agglutinin prefer Me α-Gal only by 10 and 1.8 fold respectively [36,37].

In the *A. hirsuta* lectin, hydroxyl groups at C-4, C-6 and the aglycone at C-1 in the sugar, are important. Another galactosyl lectin from tunicate *Didemnum cadidum* (DC L-1) interacts with the hydroxyls on carbons 2,3,4 of sugars. [38]. Lectins from peanut [39] and *Bandeiraea simplicifolia* I [40] interact at the C-2, 4, 6 hydroxyl groups.

The *A. hirsuta* lectin fails to bind β-linked disaccharides including the T antigen disaccharide (Gal β1-3 GalNAc) while the *A. integrifolia* lectin does not bind to lactose, LacNAc and Gal β1-3 GlcNAc but binds strongly to Gal β1-3 GalNAc [41].

In the *A. hirsuta* and *A. integrifolia* lectins, association constants for the binding of disaccharides in which galactose is in α-linkage with other sugars is significantly lower than that observed for methyl α-D galactose, suggesting that replacement of the apolar methyl group of Me α-Gal with a hydrophilic hexapyranose residue, as in Gal α1- 3 Gal, leads to diminished apolar contacts, resulting in their poor binding to the lectin. Gal α1-6 Glc (melibiose) by virtue of having a single galactopyranose residue is expected to bind through its non-reducing sugar alone suggesting that the lectins interact with these sugars through their non-reducing hexapyranose residue [41].

*A. lakoocha* lectin as reported by Wongkham et al. [10] did not react with Gal β1-3 GalNAc but is inhibited by O-glycosidically linked glycans (mucin, fetuin) which contain Gal/GalNAc in their structures. The lectin reported in this study is different from the *A. lakoocha* lectin characterised by Chatterjee et al. [4], which is inhibited by Gal β1-3 GalNAc. The differences could possibly be due to genetic polymorphism or post synthetic modification. The *A. incisa* lectin reported by Moreira et al. [7] is inhibited only by asialofetuin, suggesting a complex carbohydrate specificity involving galactose. In artocarpin, the mannose binding lectin

from *A. integrifolia* [14], Me α-Man is a marginally better ligand over mannose and glucose. The lectin recognises exquisitely the β (1-2) substituted core trimannosidic structure inclusive of the first GlcNAc residue from the non-reducing end in N-linked glycans.

Scatchard analysis using 4-methylumbelliferyl α-galactose indicated two binding sites per tetramer for the *A. hirsuta* lectin, *A. lakoocha* lectin [4] and for jacalin [16]. Upon re-determination of the $M_r$ of jacalin, the number of binding sites was changed from 2.2 to 3.3 per molecule [22]. Generally lectins from legumes have one and those from cereals (WGA), two binding sites per subunit. But two binding sites per tetramer have been reported in *Ricinus communis* [42], *Momordica charantia* [43] and *Abrus precatorius* lectins [44] similar to our results with the *A. hirsuta* lectin. The *A. hirsuta* lectin does not have an extended binding site unlike the peanut lectin [45], wheat germ agglutinin [46] or Con A [47].

Plant lectins including the *Artocarpus* lectins show a predominance of β sheet structure [7,48,49] and a saccharide induced change in ellipticity in the near UV C.D. spectrum. Although plant lectins differ in their overall tertiary structure, the amino acids that directly interact with carbohydrates are similar in that, residues possessing both hydrophobic and hydrophilic character are common. However not all lectins have the same essential residues. Chemical modification has been used for identifying the essential amino acid residues within a biologically active protein. While lysine is involved in the sugar binding and hemagglutination of galactose specific *Artocarpus hirsuta* and *Ricinus communis* [50] lectins, the modification of these residues hardly impairs the sugar-binding of Glc/Man specific lentil and pea lectin and galactose specific peanut lectin [51-53] although loss of their hemagglutination activity is reported. In Con A, lysine modification converts the tetrameric molecule into a dimeric one [54] but this is not

observed in the pea, peanut or the *A. hirsuta* lectins. Although lysine residues have been implicated in the sugar-binding activity of other lectins (pea, soybean, lentil and a form of WGA), RCA I and *A. hirsuta* are the only ones where the involvement of the positive charge of the ε-amino group has been demonstrated [50].

In the *Ricinus communis* agglutinin [50], lentil lectin [51] and in one form of WGA [55] reversible loss of agglutinating activity after acetylation of tyrosine by N-acetylimidazole has been reported. In *A. hirsuta,* N-acetylimidazole failed to inactivate the lectin but tetranitromethane inhibited agglutination, indicating the phenyl ring of tyrosine plays an important role in saccharide binding.

Chowdhury et al. [56] identified tryptophan, tyrosine and histidine residues as essential for activity of *A. lakoocha* lectin. Appukuttan and Basu [57] could not detect tryptophan in jacalin but identified lysine and tyrosine as the essential residues. Subsequently Mahanta et al. [41] by circular dichroism studies identified the presence of tryptophan in the binding site of jacalin. They proposed a model wherein subsite A in the lectin is specific for α-galactoside derivatives and has tyrosine as well as tryptophan residues while subsite B is accessible only for β-linked Gal or GalNAc residues and has only tyrosine. Tyrosine and lysine are essential for the activity of the *A. hirsuta* lectin. Although tryptophan is not involved in the saccharide binding of the lectin, ligand binding and solute quenching studies suggest that tryptophan could be situated near the saccharide binding site.

Fluorescence of methylumbelliferyl (MeUmb) glycosides is markedly influenced on binding with several proteins as a result of change in the polarity of the umbelliferyl group of the glycosides on binding to these proteins. Almost total quenching of MeUmb glycoside fluorescence is observed upon binding to *A. hirsuta* lectin similar to Con A [58], WGA

[59], *M. charantia* [43] and rice [60] lectins, suggesting that the sugar binding sites on these lectins are hydrophobic in nature. On the contrary lectins from *Pisum sativum* and *Ricinus communis* [42] do not show any change in fluorescence, indicating no change in the polarity of the environment. In *Abrus precatorius* agglutinin, partial (30%) quenching of MeUmb β-Gal fluorescence [61] and in case of soybean agglutinin 15 % enhancement in MeUmb β-GalNAc fluorescence is observed [62]. The *A. hirsuta* lectin shows no change in the fluorescence of MeUmb β-Gal whereas the umbelliferyl groups of α- and β- galactosides experience different, non-polar and polar microenvironments respectively upon binding to winged bean basic agglutinin (quenching with α-umbelliferyl galactoside and enhancement with β-umbelliferyl galactoside) [63]. *A. hirsuta* lectin shows an exothermic process similar to the lectins from *M. charantia,* wheat germ, Con A, rice and *B. simplicifolia* [43,58-60,64] for the binding of sugars.

## List of publications


1. α-Galactoside binding lectin from *Artocarpus hirsuta*: Characterisation of the sugar specificity and binding site.
   Gurjar, M.M., Khan, M.I. and Gaikwad S.M. (1998)
   Biochim. Biophys. Acta 1381, 256-264.
2. Fluorimetric studies on saccharide binding to the basic lectin from *Artocarpus hirsuta.*
   Gaikwad, S.M., Gurjar, M.M. and Khan, M.I. (1998)
   Biochemistry and Molecular Biology International (In Press)
3. Saccharide binding studies and applications of *Artocarpus* lectins.
   Gaikwad, S.M., Gurjar, M.M. and Khan, M.I. (1998)
   (Manuscript under preparation).

## SALIENT FEATURES

1. *Artocarpus hirsuta* seeds contain an α-galactoside specific lectin.
2. The lectin is heterotetrameric, glycosylated, with pI 8.5 and molecular mass 60,000.
3. C-4, C-6 and the aglycone at C-1 in the sugar are important binding loci for the lectin, which shows high affinity for Me α-Gal. Increased affinity for large hydrophobic substituents such as pNPα/β galactosides, suggests a non-polar site contiguous to the galactose-binding site.
4. The cationic charge of the ε-amino group of lysine and the phenyl ring of tyrosine are essential for saccharide binding of the lectin.
5. The lectin contains four tryptophan residues, of which one is surface localised, in the vicinity of positively charged amino acids and near the sugar-binding site but is not directly involved in sugar binding. The other three tryptophans are present in the hydrophobic core of the protein.
6. The lectin has two binding sites per molecule. It is not specific for the T antigenic disaccharide, shows low affinity for the α-linked disaccharides and does not bind blood group B antigenic trisaccharides. The driving force for the avid binding to Me α-Gal and MeUmb α-Gal is favourable entropy.
7. 35 % of the activity of the seed extract is adsorbed on DEAE-cellulose during purification. This is due to the association of an additional mannose binding polypeptide which confers acidic nature to the lectin. Pre-incubation with mannose followed by affinity chromatography removes this impurity yielding the basic lectin (AppendixⅡ).
8. The immobilised lectin can be used for the separation/ purification of mucins and polysaccharides containing α-galactose (Appendix Ⅰ).

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# APPENDIX I

# AFFINITY CHROMATOGRAPHY ON IMMOBILISED *ARTOCARPUS HIRSUTA* LECTIN

## INTRODUCTION

The role of glycoconjugates has been demonstrated in many biological interactions. Hence it is important to purify and analyse their structures. This can be effectively done by affinity chromatography on immobilised lectins. The basis of lectin affinity chromatography is that lectins interact with high affinity with certain types of oligosaccharides and can also distinguish complex structural features of glycoconjugates. Lectin affinity chromatography is relatively easy and when used in combination with other techniques like radiolabelling permits the purification of small amounts of material. Although definite deductions about structure cannot be obtained using one immobilised lectin, it can be achieved using a series of different immobilised lectins (serial lectin affinity chromatography) [1]. For this purpose lectins that recognise features of the core structure are utilised initially and lectins recognising the more peripheral sugars are used later.

In nature, polysaccharides are often found mixed with proteins, nucleic acids and other tissue components. Since separation of polysaccharides by solvent precipitation also precipitates extraneous matter simultaneously, their purification by affinity chromatography on immobilised lectins is a viable alternative. Separation and purification of proteins having N- or O-linked oligosaccharides is another area where immobilised lectins can be effectively used because of their selective binding affinity. Among *Artocarpus* lectins, *Artocarpus integrifolia* lectin immobilised on agarose and Sepharose has been used for the separation of proteins with O-linked oligosaccharides and for the purification of galactomannans [2,3].

Hence, attempts were made to immobilise the *A. hirsuta* lectin onto Sepharose 4B and evaluate its potential as an analytical tool.

## MATERIAL AND METHODS

### *Materials*

Sepharose was purchased from Pharmacia (Uppasala, Sweden). Divinyl sulfone, gums and glycoproteins were obtained from Sigma Chemical Company (St. Louis, U.S.A.). All other chemicals used were of analytical grade. The basic lectin from *Artocarpus hirsuta* was purified as described in chapter II.

### *Methods*

**Determination of protein**: Protein was estimated according to the method of Lowry et al. [4] using bovine serum albumin as standard.

**Preparation of *A. hirsuta* lectin—Sepharose**: Sepharose 4B was successively washed with distilled water (to remove the preservatives), 200 mM sodium carbonate buffer, pH 10, (till the filtrate was of pH 10). 10 ml (packed volume) of Sepharose-4B was suspended in 10 ml $Na_2CO_3$ (1M), containing 2 ml divinyl sulfone and incubated at room temperature (27°C) for 2 h under mild agitation. Excess divinyl sulfone was removed by extensive washing with sodium carbonate buffer and distilled water. Coupling of *A. hirsuta* lectin to the Sepharose was carried out by reacting the activated matrix with 10 mg of the lectin (in 1 ml of 100 mM $NaHCO_3$ containing 500 mM NaCl), for 2 h at room temperature (27°C) under mild agitation. The lectin-Sepharose mixture was filtered and washed several times with 50 mM phosphate buffer, pH 8.0 containing 500 mM NaCl. The amount of lectin bound to the matrix was determined by estimating the amount of protein before and after coupling. The affinity matrix prepared by this method contained 1mg lectin/ml of Sepharose. The matrix was

stored, in wet state, at pH 7.5 and at 4°C in the presence of 0.02 % sodium azide.

**Affinity Chromatography**: Unless otherwise stated, all operations were carried out at 4±1°C. During the purification steps, the protein concentrations were monitored by measuring absorbance at 280 nm, while that of gums and mucins by the phenol sulfuric acid method of Dubois [5] using galactose as standard. Desialytion of mucins was carried out by incubation with neuraminidase (1 unit per 10 mg mucin) at pH 5.0 and at 37°C for 2 h, followed by extensive dialysis against THS.

Different glycoproteins and gums were adsorbed onto the *A. hirsuta* lectin–Sepharose column (1 cm × 10 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl (THS). The column was washed with THS till the effluent showed no protein or gum. Subsequently, the bound mucins were eluted by washing the column with tris buffered saline, pH 8.8. The bound polysaccharides were eluted with 300 mM galactose in THS pH 7.0. Fractions of 1.5 ml were collected at a rate of 12ml/h.

## RESULTS AND DISCUSSION

Lectins due to their varied sugar specificity have found extensive application in the determination of carbohydrate structure, detecting cell surface changes during development, differentiation, malignancy and in the isolation of a number of biomolecules. *A. hirsuta* lectin described in the present work shows specificity for α-galactose residues in the carbohydrate structure. The lectin was immobilised on Sepharose and used for the separation of glycoproteins and polysaccharides.

Among the glycoproteins tested ovalbumin, fetuin, fibrinogen and orosomucoid were not retained on the lectin affinity matrix (Fig. App.1). The inability of the matrix to retain ovalbumin can be correlated to its high mannose content. The lack of affinity of the other glycoproteins tested can be due to the presence of the disaccharide Gal β1-3 GlcNAc [6,7] to which the *A. hirsuta* lectin is not complementary (Chapter IV).

Pig submaxillary mucin (PSM) and bovine submaxillary mucin (BSM) were retained on the column (Fig. App.1). The non-specific binding of mucins due to their viscous nature and acidic charge was ruled out by chromatography of desialyted mucins and it was noted that the desialyted forms could also bind to the affinity matrix. A mucin molecule may be perceived as a long polypeptide backbone carrying numerous O-glycosidally linked sugar chains [8]. The retention of mucins is indicative of the fact that the lectin recognises terminal or subterminal α-GalNAc.

Therefore the above results suggest that immobilised *A. hirsuta* lectin can be used for the separation/ purification of certain glycoproteins bearing O-linked oligosaccharides like mucins. Most of the N-linked oligosaccharides in glycoproteins (transferrin, $\alpha_1$ acid glycoprotein) do not contain galactose or N-acetyl galactosamine in α-anomeric linkages whereas the *A. hirsuta* lectin is specific only for α-galactosides.

Chromatography of gums on the lectin affinity matrix revealed that except for gum ghatti and arabinogalactan, all the other gums tested showed significant retention (Table I). Larch arabinogalactan has a β-D(1-3) linked galactan framework, with branches of β-D(1-6) linked galactose residues. Arabinose residues also occur as endgroups [9]. Gum ghatti is a glucuronomannan containing arabinose and glucuronic acid as end groups, a small proportion of β-D galactopyranosyl residues and core residues of

mannose [10]. Since *A. hirsuta* lectin shows high affinity for terminal α-galactose, the very poor affinity of these two can be expected.

Locust bean gum from the seeds of *Ceratonia siliqua*, contains α-D galactopyranosyl side-groups disposed in uniform blocks along the backbone of (1-4) linked mannopyranose residues. In guar gum the α-galactose side-chain units are alternately disposed along the mannan backbone [11]. Both guar gum and locust bean gum showed high affinity towards the immobilised lectin.

Karaya gum, the exudate gum from *Sterculia urens* is a partially acetylated polymer mainly made up of alternating residues of D-galacturonic acid and L-rhamnose in the interior chains with side chains terminating in D-glucuronic acid as well as D-Gal residues. As *A. hirsuta* lectin has no affinity towards the former, the decreased retention of karaya gum can be correlated to the presence of glucuronic acid in the terminal position [12]. Gum arabic, contains D-galactose residues in different locations. The β-D-galactan core consists of relatively uniform subunits of ~13 (1-3) linked residues to which other β-D galactose residues are attached in sidechains. Other sugar residues, notably L-arabinose, D-glucuronic acid and L-rhamnose are in the outer chains together with those of α-D-galactopyranose. These latter residues occupy terminal positions in the polysaccharide structure. The α-D-galactopyranose residue present in gum arabic might be contributing to its affinity for immobilised *A. hirsuta* lectin [13]. Compared to guar gum and locust bean gum, the relatively lower affinity of gum arabic and karaya gum for the immobilised lectin could be due to the presence of varying terminal sugar residues.

Galacturonorhamnans bind to the immobilised *A. hirsuta* lectin due to the terminal α-galactose residue. In galactomannans having α-D-Gal 1,6-linkage (like guar gum and locust bean gum containing non-reducing

terminal D-Gal residues, 1,6 glycosidically linked to the mannan backbone), there is flexibility for rotation about 1,6 glycosidic bonds which facilitates the greater reactivity of these polysaccharides on the basis of the methylene group (the methylene group at C-6 of the reducing sugar is topographically similar to that of the methyl group present in methyl α-galactose). For this reason plant galactomannans are almost totally retained on the affinity matrix [14].

Although *A. hirsuta* lectin lacks an extended binding site, submaxillary mucins bind the immobilised lectin due to a cluster effect. α-galactose present at their chain terminal binds the *A. hirsuta* lectin and one mucin molecule reacts with more than one lectin binding site. A similar observation of co-operative effect has been made in *A. integrifolia* lectin which binds asialoglycophorin A having fifteen O-linked oligosaccharide chains and one N-linked oligosaccharide [15].

In conclusion, the immobilised *A. hirsuta* lectin can be used for the separation of peptidoglycans containing O-linked saccharides and plant polysaccharides containing terminal α-galactose residues.

**Table I: Gums fractionated on immobilised *A. hirsuta* lectin.**

| Gum | Fraction<br>U: unbound<br>B: bound | % binding to column<br>(Neutral Sugar) |
|---|---|---|
| Guar | U | 3.7 |
| | B | 96.3 |
| Locust | U | 4.9 |
| | B | 95.1 |
| Karaya | U | 25 |
| | B | 75 |
| Arabic | U | 20 |
| | B | 80 |
| Arabinogalactan | U | 100 |
| | B | 0 |
| Ghatti | U | 83 |
| | B | 17 |

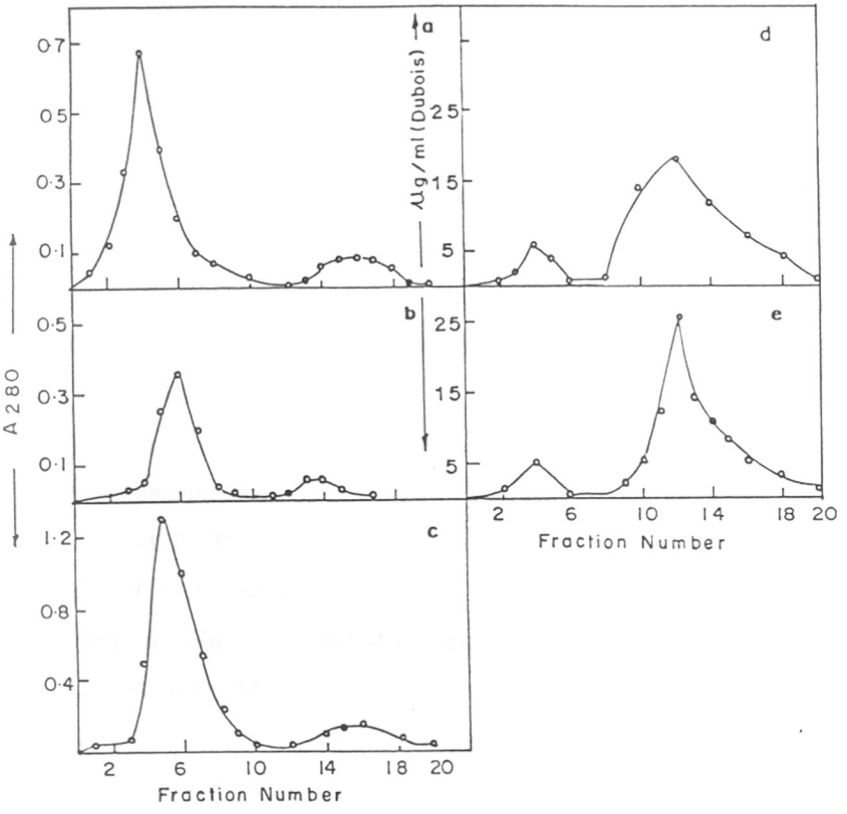


**Fig. App.1:**

**Elution profiles of glycoproteins on the *A. hirsuta* lectin-Separose 4B column: a) fetuin, b) orosomucoid c) transferrin d) desialyted pig submaxillary mucin e) desialyted bovine submaxillary mucin**

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# APPENDIX II

# CHARACTERISATION OF A VARIANT LECTIN FROM *ARTOCARPUS HIRSUTA*

Presence of both acidic and basic lectins have been reported in winged bean [1]. During the purification of the basic lectin, it was observed that approximately 30-40% of the hemagglutinating activity, found in the seed extract of *Artocarpus hirsuta,* was retained on DEAE-cellulose. The bound protein could agglutinate erythrocytes in high titre. Hence purification and characterisation of this lectin was carried out.

The lectin was purified to homogeneity by chromatography on DEAE-cellulose (pH 7.2) followed by affinity chromatography on cross-linked guar gum. $M_r$ of the lectin determined by gel filtration on Sephadex G-100 was 72,000 and it consisted of three non-identical subunits of $M_r$ 20,000, 15,800 and 14,200. (The major lectin from *A. hirsuta,* described in Chapter II, is a heterotetramer of non-identical subunits with $M_r$ 15,800 and 14,200. An additional subunit of 20,000 was detected in the present lectin). Isoelectric focussing of this lectin on polyacrylamide gel (pH 3-10) yielded two bands with pI values of 8.5 (pI of the major lectin) and 6.0.

Among the monosaccharides, galactose was a better inhibitor of the variant *A. hirsuta* lectin. The hydroxyl groups at C-4 and C-6 were critical for binding, as glucose, mannose and fucose were non-inhibitory. Methyl α-galactose was 40 fold more effective as an inhibitor compared to methyl β-galactose. The sugar specificity of this lectin was similar to that of the major lectin described in Chapter II.

Modification of the lectin by DEP resulted in complete loss of activity with concomitant modification of one histidine residue per molecule of the lectin. However, there was no reversal of activity of the DEP inactivated lectin by hydroxylamine hydrochloride.

Citraconylation of the lectin revealed the involvement of four lysine residues per molecule in the lectin activity. Decitraconylation at pH 4.0 could restore the activity completely in 1 h. Moreover Me α-Gal (the

inhibitory sugar) offered complete protection against citraconic anhydride mediated inactivation confirming the involvement of lysine in the lectin activity.

Nitration of the lectin with TNM caused 90% inactivation with modification of four tyrosine residues and Me α-Gal could protect the lectin from TNM mediated inactivation. Inactivation of this lectin by TNM indicated that the phenyl ring of tyrosine could be involved in the sugar binding.

Chemical modification studies also revealed that arginine, serine, carboxyl and tryptophan residues have no role in the hemagglutinating activity of the lectin. The residues involved in the binding site of this lectin were same as those found in the lectin described in the preceding chapters.

The sugar specificity and amino acid residues present at the sugar-binding site were similar for both the lectins. Two of the three subunits of the lectin described here and one of its isoforms was similar to the lectin described in the Chapters II and III. Therefore the different chromatographic behaviour of the lectin described here, on DEAE-cellulose, could be because of a prominent surface negative charge due to the additional subunit. An earlier report on jacalin showed the presence of 35 bands on isoelectric focussing, in a broad pH range from acidic to alkaline (5-8.5) [2], which led us to believe that *A. hirsuta* seeds also possess acidic and basic lectins.

*A. integrifolia* seeds possess in addition to jacalin, a second lectin of different sugar specificity (mannose). In-order to determine whether the *A. hirsuta* lectin described here also has specificity for mannose, which may be masked by the major lectin, it was pre-incubated with mannose and then subjected to guar gum affinity chromatography. The galactose eluted lectin was identical to the major lectin described in the preceding chapters in its

physico-chemical properties, suggesting that both the lectins i.e. DEAE-cellulose bound and unbound lectins are the same. Moreover the flow through fractions obtained from guar gum affinity chromatography (with mannose pre-incubation), were not inhibited by mannose or its derivatives as checked by hemagglutination inhibition. Hence the adhering polypeptide, released in the presence of mannose is not an agglutinin but could possibly be the unprocessed α subunit or some other mannose binding protein.

The 35% activity in the seed extract that bound to DEAE-cellulose was in fact the major lectin whose behaviour on the ion exchanger was altered due to the strongly adhering polypeptide that probably binds to the high mannose oligosaccharide of the original lectin. The above studies suggest that the seeds of *A. hirsuta* contain only one galactose binding lectin.

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